Exhibit G
Application No.08/041,431

YEAST-BASED PEPTIDE LIBRARIES FOR IDENTIFICATION OF G PROTEIN-COUPLED RECEPTOR AGONISTS AND ANTAGONISTS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the screening of random peptides in yeast cells for the ability to interact with cellular signal transduction pathways.

Description of the Background Art

Drug: Receptor interactions as transducible signals.

For a drug to cure a disease or alleviate its symptoms, the drug must be delivered to the appropriate cells, and trigger the proper "switches." The cellular switches are known as "receptors." Hormones, growth factors, neurotransmitters and many other biomolecules normally act through interaction with specific cellular receptors. Drugs may activate or block particular receptors to achieve a desired pharmaceutical effect.

Cell surface receptors mediate the transduction of an "external" signal (the binding of a ligand to the receptor) into an "internal" signal (the modulation of a cytoplasmic metabolic pathway).

In many cases, transduction is accomplished by the following signaling cascade:

- An agonist (the ligand) binds to a specific protein (the receptor) on the cell surface.

- As a result of the ligand binding, the receptor undergoes an allosteric change which activates a transducing protein in the cell membrane.

- The transducing protein activates, within the cell, production of so-called "second messenger molecules."

- The second messenger molecules activate certain regulatory proteins within the cell that have the potential to "switch on" or "off" specific genes or alter some metabolic process.

This series of events is coupled in a specific fashion for each possible cellular response. The response to a specific ligand may depend upon which receptor a cell expresses. For instance, the response to adrenalin in cells expressing α-adrenergic receptors may be the opposite of the response in cells expressing β-adrenergic receptors.

The above "cascade" is idealized, and variations on this theme occur. For example, a receptor may act as its own transducing protein, or a transducing protein may act directly on an intracellular target without mediation by a "second messenger".

One family of signal transduction cascades found in eukaryotic cells utilizes heterotrimeric "G-proteins." Many different G proteins are known to interact with receptors. G-protein signaling systems include three components: the receptor itself, a GTP-binding protein (G-protein), and an intra-cellular target protein.

The cell membrane acts as a switchboard. Messages arriving through different receptors can produce a single effect if the receptors act on the same type of G protein. On the other hand, signals activating a single receptor can produce more than one effect if the receptor acts on different kinds of G proteins, or if the G proteins can act on different effectors.

In their resting state, the G-proteins, which consist of alpha (α), beta (β) and gamma (γ) subunits, are complexed with the nucleotide guanosine diphosphate (GDP) and are in contact with receptors. When a hormone or other first messenger binds to receptor, the receptor changes conformation and this alters its interaction with the G-protein. This spurs the alpha subunit to release GDP, and the more abundant nucleotide guanosine triphosphate (GTP), replaces it, activating the G-protein. The G-protein then dissociates to separate the alpha subunit from the still complexed beta and gamma subunits. Either the Gα subunit, or the G$\beta\gamma$ complex, depending on the pathway, interacts with an effector. The effector (which is often an enzyme) in turn converts an inactive precursor molecule into an active "second messenger," which may diffuse through the cytoplasm, triggering a metabolic cascade. After a few seconds, the Gα converts the GTP to GDP, thereby inactivating itself. The inactivated Gα may then reassociate with the G$\beta\gamma$ complex.

Hundreds, if not thousands, of receptors convey messages through heterotrimeric G-proteins, of which at least 17 distinct forms have been isolated. Although the greatest variability has been seen in the α subunit, several different β and γ structures have been reported. There are, additionally, several different G protein-dependent effectors.

Most G-protein coupled receptors are comprised of a single protein chain that is threaded through the plasma membrane seven times. Such receptors are often referred to as seven-transmembrane receptors (STRs). More than a hundred different STRs have been found, including many distinct receptors that bind the same ligand, and there are likely many more STRs awaiting discovery.

Yeast Pheromone G Protein Mediated Signal Pathways

Haploid yeast cells are able to grow vegetatively, or to mate to form a diploid cell. The two mating types ("sexes") of haploid cells are designated **a** and α. The **a** cells produce the

a factor, and the α cells, the α factor. These are called pheromones, because the yeast cells of the opposite sex respond to them. Thus, **a** cells bear α-factor receptors, and α cells bear **a** factor receptor. Both the **a** factor and the α factor receptors are G protein-coupled STRs.

In normal S. cerevisiae (budding yeast) **a** cells, the α factor binds the receptor STE2. The G protein dissociates, and the Gβγ binds an unidentified effector, which in turn activates a number of genes. STE20, a kinase, activates STE5, a protein of unknown function. STE5 activates STE11 kinase, which stimulates STE7 kinase, which spurs into action the KSS1 and/or FUS3 kinases. These switch on expression of the transcription activator STE12. STE12 stimulates expression of a wide variety of genes involved in mating, including FUS1 (cell fusion), FAR1 (cell-cycle arrest), STE2 (the receptor), MFA1 (the pheromone), SST2 (recovery), KAR3 (nuclear fusion) and STE6 (pheromone secretion). Other genes activated by the pathway are CHS1, AGα1, and KAR3. The multiply tandem sequence TGAAACA has been recognized as a "pheromone response element" found in the 5'-flanking regions of many of the genes of this pathway.

One of the responses to mating pheromone is the transient arrest of the yeast cell in the G1 phase of the cell cycle. This requires that all three G1 cyclins (CLN1, CLN2, CLN3) be inactivated. It is believed that FUS3 inactivates CLN3, and FAR1 deals with CLN2. (The product responsible for inactivating CLN1 is unknown).

The growth arrest is terminated by a number of different mechanisms. First, the α-factor receptor is internalized following binding of the pheromone, resulting in a transient decrease in the number of pheromone binding sites. Second, the C-terminal tail of the receptor is phosphorylated consequent to ligand binding, resulting in uncoupling of the receptor from the transducing G proteins. Third, pheromone-induced increases in expression of GPA1p (the Gα-subunit of the heterotrimeric G protein) increase the level of the subunit relative to the and

subunits, resulting in reduction in the level of free and consequent inactivation of the pheromone response pathway. Additional mechanisms include induction of the expression of SST2 and BAR1 and phosphorylation of the subunit (perhaps by SVG1).

Signaling is inhibited by expression of a number of genes, including CDC36, CDC39, CDC72, CDC73, and SRM1. Inactivation of these genes leads to activation of the signalling pathway.

A similar pheromone signaling pathway may be discerned in α cells, but the nomenclature is different in some cases (e.g., STE3 instead of STE2).

Other yeast also have G protein-mediated mating factor response pathways. For example, in the fission yeast S. pombe, the M factor binds the MAP3 receptor, or the P-factor the MAM2 receptor. The dissociation of the G protein activates a kinases cascade (BYR2, BYR1, SPK1), which in turn stimulates a transcription factor (STE11). However, in S. pombe, the Gα subunit transmits the signal, and there are of course other differences in detail.

An **a** cell may be engineered to produce the **a** factor receptor, and an α cell to make α factor receptor. Nakayama, et al., EMBO J., 6:249-54 (1987); Bender and Sprague, Jr., Genetics 121: 463-76 (1989).

Heterologous receptors have been functionally expressed in S. cerevisiae. Marsh and Hershkowitz, Cold Spring Harbor Symp., Guant. Biol., 53: 557-65 (1988) replaced the S. cerevisiae STE2 with its homologue from S. Kluyven. More dramatically, a mammalian beta-adrenergic receptor and Gα subunit have been expressed in yeast and found to control the yeast mating signal pathway. King, et al., Science, 250: 121-123 (1990).

The effects of spontaneous and induced mutations in pheromone pathway genes have been studied. These include the α-factor (MFα1 and MFα2) genes, see Kurjan, Mol. Cell. Biol., 5:787

(1985); the a-factor (MFa1 and MFa2) genes, see Michaelis and Herskowitz, Mol. Cell. Biol. 8:1309 (1988); the pheromone receptor (STE2 and STE3) genes, see Mackay and Manney, Genetics, 76:273 (1974), Hartwell, J. Cell. Biol., 85:811 (1980), Hagen, et al., P.N.A.S. (USA), 83:1418 (1986); the FAR1 gene, see Chang and Herskowitz, Cell, 63:999 (1990); and the SST2 gene, see Chan and Otte, Mol. Cell. Biol., 2:11 (1982).

Yeast strains that are auxotrophic for histidine (HIS3) are known, see Struhl and Hill), Mol. Cell. Biol., 7:104 (1987); Fasullo and Davis, Mol. Cell. Biol., 8:4370 (1988). The HIS3 (imadezoleglycerol phosphate dehydratase) gene has been used as a selective marker in yeast. See Sikorski and Heiter, Genetics, 122:19 (1989); Struhl, et al., P.N.A.S. 76:1035 (1979); and, for FUS1-HIS3 fusions, see Stevenson, et al., Genes Dev., 6:1293 (1992).

Duke University, WO92/05244 (April 2, 1992) describes a transformed yeast cell which is incapable of producing a yeast G protein alpha subunit, but which has been engineered to produce both a mammalian G protein alpha subunit and a mammalian receptor which is "coupled to" (i.e., interacts with) the aforementioned mammalian G protein alpha subunit. Specifically, Duke reports expression of the human beta-2 adrenergic receptor (hβAR), an STR, in yeast, under control of the GAL1 promoter, with the hβAR gene modified by replacing the first 63 base pairs of noncoding and 42 base pairs of coding sequence from the STE2 gene. (STE2 encodes the yeast α-factor receptor). Duke found that the modified hβAR was functionally integrated into the membrane, as shown by studies of the ability of isolated membranes to interact properly with various known agonists and antagonists of hβAR. The ligand binding affinity for yeast-expressed hβAR was said to be nearly identical to that observed for naturally produced hβAR.

Duke co-expressed a rat G protein alpha subunit in the same cells, yeast strain 8C, which lacks the cognate yeast protein. Ligand binding resulted in G protein-mediated signal transduction.

Duke teaches that these cells may be used in screening compounds for the ability to affect the rate of dissociation of $G\alpha$ from $G\beta\gamma$ in a cell. For this purpose, the cell further contains a pheromone-responsive promoter (e.g. BAR1 or FUS1), linked to an indicator gene (e.g. HIS3 or LacZ). The cells are placed in multi-titer plates, and different compounds are placed in each well. The colonies are then scored for expression of the indicator gene.

Duke's yeast cells do not, however, actually produce the compounds to be screened. As a result, only a relatively small number of compounds can be screened, since the scientist must make sure that a given group of cells is contacted with only a single, known compound.

Peptide Libraries

Peptide libraries are systems which simultaneously display, in a form which permits interaction with a target, a highly diverse and numerous collection of peptides. These peptides may be presented in solution (Houghten), or on beads (Lam), chips (Fodor), bacteria (Ladner), spores (Ladner), plasmids (Cull) or on phage (Scott, Devlin, Cwirla, Felici, Ladner). Many of these systems are limited in terms of the maximum length of the peptide or the composition of the peptide (e.g., Cys excluded). Steric factors, such as the proximity of a support, may interfere with binding. The screening is for binding in vitro to an artificially presented target, not for activation or inhibition of a cellular signal transduction pathway in a living cell. While a cell surface receptor may be used as a target, the screening will not reveal whether the binding of the peptide caused an allosteric change in the conformation of the receptor.

Ladner, USP 5,096,815 describes a method of identifying novel proteins or polypeptides with a desired DNA binding activity. Semi-random ("variegated") DNA encoding a large number of different potential binding proteins is introduced, in

expressible form, into suitable host cells. The target DNA sequence is incorporated into a genetically engineered operon such that the binding of the protein or polypeptide will prevent expression of a gene product that is deleterious to the gene under selective conditions. Cells which survive the selective conditions are thus cells which express a protein which binds the target DNA. While it is taught that yeast cells may be used for testing, bacterial cells are preferred. The interactions between the protein and the target DNA occur only in the cell, not in the periplasm, and the target is a nucleic acid, and not a transmembrane receptor protein.

All references cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art.

SUMMARY OF THE INVENTION

The present invention relates to a method of assaying potential peptides for the ability to interact with a receptor of interest. In this invention, yeast cells are engineered, first, to graft a receptor of interest into the yeast pheromone signal transduction pathway in such a way that the interaction of a peptide with that receptor is detectable by screening or selection of the yeast cells, and second, so that the yeast cells engineered to provide the receptor also express the peptide library to be assayed.

While others have engineered yeast cells to facilitate screening of exogenous receptor agonists and antagonists, the cells did not themselves produce the drugs. The engineered yeast cells that do not produce the drugs themselves are inefficient. To utilize them one must bring a sufficient concentration of each drug into contact with a number of cells in order to detect whether or not the drug has an action. Therefore, a microliter plate well or test tube must be used for each drug. The drug must be synthesized in advance and be sufficiently pure to judge its action of the yeast cells. However, the present invention

allows for the testing of 10E7 to 10E8 or more peptide members, i.e. MegaLibraries.

Moreover, in a preferred embodiment of the present invention, the assay is designed so that yeast cells which express peptides having the desired activity can be selected, and not merely identified.

These peptide drugs may be agonists or antagonists of the ligands which normally interact with the receptor. An agonist is a ligand which binds to the receptor, and activates its signal transduction pathway (e.g., by causing an allosteric change in the receptor). An antagonist is a ligand which binds to receptor, but does not activate it. It does, however, block the binding site of the receptor so that it is no longer accessible to agonists.

As previously mentioned, one of the consequences of activation of the pheromone signal pathway in wild-type yeast is growth arrest. If one is testing for antagonist activity, this normal response of growth arrest can be used to select cells in which the pheromone response pathway is inhibited. That is, cells exposed to both a known agonist and a peptide of unknown activity will be growth arrested if the peptide is neutral or an agonist, but will grow normally if the peptide is an antagonist. Thus, the growth arrest response can be used to advantage to discover peptides that function as antagonists.

However, when searching for peptides which can function as agonists, the growth arrest consequent to activation of the pheromone response pathway is an undesirable effect for this reason: cells that bind peptide agonists stop growing while surrounding cells that fail to bind peptides will continue to grow. The cells of interest, the, will be overgrown or their detection obscured by the background cells, confounding identification of the cells of interest. To overcome this problem the present invention teaches engineering the cell such that: 1) growth arrest does not occur as a result of pheromone

signal pathway activation (e.g., by inactivating the FAR1 gene); and/or 2) a selective growth advantage is conferred by activating the pathway (e.g., by transforming an auxotrophic mutant with a HIS3 gene under the control of a pheromone-responsive promoter, and applying selective conditions).

- It is, of course, desirable that the exogenous receptor be exposed on a continuing basis to the peptides. Unfortunately, this is likely to result in desensitization of the pheromone pathway to the stimulus. Desensitization may be avoided by mutating (which may include deleting) the SST2 gene so that it no longer produces a functional protein, or by mutating other genes which may contribute to desensitization, e.g., BAR1 in the case of **a** cells and SVG1 for either **a** or α cells.

If the endogenous pheromone receptor is produced by the yeast cell, the assay will not be able to distinguish between peptides which bind the pheromone receptor and those which bind the exogenous receptor. It is therefore desirable that the endogenous gene be deleted or otherwise rendered nonfunctional.

In a preferred embodiment, at least some peptides of the peptide library are secreted into the periplasm, where they may interact with the "extracellular" binding site(s) of the exogenous receptor. They thus mimic more closely the clinical interaction of drugs with cellular receptors. This embodiment optionally may be further improved by preventing pheromone secretion, and thereby avoiding competition between the peptide and the pheromone for signal peptidase and other components of the secretion system.

The claims are hereby incorporated by reference as a further description of the preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Outline of successive stages in the developm nt of yeast aut crine systems.

An outline of the normal synthesis and release of mating pheromones is diagrammed in th upper left. Two g nes, *MFα1* and *MFα2*, encode precursor proteins (MFα1p and MFα2p) containing four and two repeats, respectively, of the tridecapeptide representing mature α-factor. These precursors are processed proteolytically in a series of enzymatic reactions that begin with cleavage of the signal sequence in the endoplasmic reticulum and involve both glycosylation of the leader peptide and cleavage by the proteases KEX2p, STE13p, and KEX1P. The result is the secretion of mature pheromone which, upon binding to STE2p normally expressed on the surface of **a** cells, elicits a number of changes in the **a** cells, including growth arrest. The **a** cells, in turn, express two genes, *MFa1* and *MFa2*, which encode precursors (MFa1p and MFa2p) for **a**-factor. These precursors undergo farnesylation by RAM1 and RAM2, proteolytic trimming of the C-terminal three amino acids (by a protein tentatively identified as RAM3p), carboxymethylation of the newly exposed C-terminal cysteine by STE14p, and endoproteolytic removal of the N-terminal leader sequence by an activity provisionally identified as STE19p. Upon export of the mature **a**-factor from the cell via STE6p, it binds to STE3p expressed on the surface of α cells and stops their growth.

Stage 1 involves the development of yeast strains in which *SST2*, *FAR1*, and *HIS3* are inactivated and a suitable reporter construct like fus1::HIS3 is integrated into the genomes of both α and **a** cells. Alpha cells are further altered by replacement of the normally expressed STE3p with STE2p, while **a** cells are further modified by replacement of the normally expressed STE2p with STE3p. The resulting strains should show growth on histidine-deficient media in the absence of exogenous pheromone.

Stage 2 involves, first, inactivation of *MFα1* and *MFα2* in cells and inactivation of MFa1 and *MFa2* in **a** cells developed in Stage 1. These modifications will result in strains which are auxotrophic for histidine. Next, the appropriate expression plasmid will be introduced: the expression plasmid pADC-MF (see Figure 4) containing an oligonucleotide encoding α-factor should

confer upon α cells the ability to grow on histidine-deficient media; th expression plasmid pADC-MFa (see Figure 6) containing an oligonucleotide encoding a-factor should enable a cells to grow on histidine-deficient media.

Stage 3 uses the cells developed in Stage 2 for the insertion of expression plasmids. However, instead of using plasmids which contain oligonucleotides that encode genuine pheromone, the yeast will be transformed with expression plasmids that contain random or semi-random oligonucleotides. Transformants which can grow on histidine-deficient media will be expanded and their plasmids isolated for sequencing the inserted oligonucleotide.

Figure 2. Diagram of the plasmid used for mutagenesis of *MFα1*. A 1.8 kb EcoRI fragment containing *MFα1* is cloned into the EcoRI site of pALTER such that single-stranded DNA containing the *MFα1* minus strand can be synthesized. The diagram illustrates the different regions of *MF1*, including the promoter, transcription terminator, and different domains of the precursor protein: the signal peptide, the pro peptide, the four repeats of mature α-factor, and the three spacers which separate these repeats. Above the block diagram of the regions of *MFα1* are the amino acid sequences of the signal peptide and the pro peptide; below it are those of the pheromone repeats and the spacers. The sites of proteolytic processing of the precursor protein are indicated by arrows, with each proteolytic activity represented by a different arrow, as indicated in the figure.

Figure 3. Diagram of the plasmids used in the construction of the MFα expression cassette. pAAH5 contains the ADC1 promoter which will be used to drive expression of synthetic oligonucleotides inserted into the MFα expression cassette. The 1.5 kb BamHI to HIndIII fragment containing the ADC1 promoter will be cloned into pRS426, a plasmid which functions as a high-copy episome in yeast, to yield pRS-ADC. pRS-ADC will be the recipient of MFα1 sequences which have been mutated as follows: The region of *MFα1* which encodes mature α-factor will be replaced with restriction sites that can accept oligonucleotides with Afl

II and Bgl II ends. Insertion of oligonucleotides with Afl II and Bgl II ends will yield a plasmid which encodes a protein containing th MFα1 signal and lead r sequences upstream of the sequence encoded by the oligonucleotide. The MFα1 signal and leader sequences should direct the processing of this precursor protein through the pathway normally used for the secretion of mature α-factor.

Figure 4. Diagram of constructs used for the expression of random oligonucleotides in the context of MFα1. Oligonucleotides containing a region of 39 random base pairs (shown at the top of the figure) will be cloned into the Afl II and Bgl II sites of the MFα1 expression cassette. These oligonucleotides will encode the six amino acids immediately N-terminal to the first repeat of the α-factor in *MFα1*, followed in succession by a tridecapeptide of random sequence and a stop codon. Yeast transformed with these constructs and selected for ability to grow on media deficient in uracil will use the ADC1 promoter to express a protein consisting of the MFα1 leader (both pre and pro peptides) followed by 13 random amino acids. Processing of the leader sequences will result in secretion of the tridecapeptide.

Figure 5. Diagram of the plasmid used for mutagenesis of *MFa1*. A 1.6 kb BamHI fragment containing *MFa1* is cloned into the BamHI site of pALTER such that single-stranded DNA containing the *MFa1* minus strand can be synthesized. The diagram illustrates the different regions of *MFa1*, including the promoter, transcription terminator, and different domains of the precursor protein: the leader peptide; the dodecapeptide that represents the peptide component of mature a-factor and whose C-terminal cysteine becomes farnesylated and carboxymethylated during processing; and the C-terminal three amino acids that are removed during processing of the precursor. Above the block diagram of the regions of *MFa1* is the amino acid sequence of the primary translation product.

Figure 6. Diagram of c nstructs used for the expression of random oligonucleotides in th context of MFa1. Oligonucleotides

containing a region of 33 random base pairs (shown at the top of the figure) will be cloned into the Xho I and Afl II sites of th MFa1 expression cass tte. These oligonucleotides will encode the seven amino acids immediately N-terminal to the first amino acid of mature a-factor, followed in succession by a monodecapeptide of random sequence, a cysteine which is farnesylated and carboxymethylated during processing of the precursor, three amino acids (VIA) which are proteolytically removed during processing, and a stop codon. Yeast transformed with these constructs and selected for ability to grow on media deficient in uracil will use the ADC1 promoter to express a precursor protein consisting of the MFa1 leader followed by 11 random amino acids and a C-terminal tetrapeptide CVIA. Processing of this precursor will result in secretion of a C-terminally farnesylated, carboxymethylated dodecapeptide which consists of 11 random amino acids and a C-terminal cysteine.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention contemplates the assaying of peptide libraries, expressed in genetically engineered yeast cells, for the ability of the peptides to interact with exogenous receptors borne by those yeast cells.

Exogenous receptors

The "exogenous receptors" of the present invention may be any G protein-coupled receptor which is exogenous to the wild-type yeast cell which is to be genetically engineered for the purpose of the present invention. This receptor may be a plant or animal cell receptor. Screening for binding to plant cell receptors may be useful in the development of, e.g., herbicides. In the case of an animal receptor, it may be of invertebrate or vertebrate origin. If an invertebrate receptor, an insect receptor is preferred, and would facilitate development of insecticides. The receptor may also be a vertebrate, more preferably a mammalian, still more preferably a human, receptor.

The exogenous receptor is also preferably a seven transmembrane segment r ceptor.

Suitable receptors include, but are not limited to, dopaminergic, muscarinic cholinergic, alpha-adrenergic, beta-adrenergic, opioid (including delta and mu), cannabinoid, serotoninergic, and GABAergic receptors. The term "receptor," as used herein, encompasses both naturally occurring and mutant receptors.

Many of these G protein-coupled receptors, like the yeast **a** and α factor receptors, contain seven hydrophobic amino acid-rich regions which are assumed to lie within the plasma membrane. Specific human G protein-coupled STRs for which genes have been isolated or could be constructed include those listed in Table 1. Thus, the gene would be operably linked to a promoter functional in yeast and to a signal sequence functional in yeast. Suitable promoters include Ste2, Ste3 and gal10. Suitable signal sequences include those of Ste2, Ste3 and of other genes which encode proteins secreted by yeast cells. Preferably, the codons of the gene would be optimized for expression in yeast. See Hoekema et al., Mol. Cell. Biol., 7:2914-24 (1987); Sharp, et al., 14:5125-43 (1986).

The homology of STRs is discussed in Dohlman et al., Ann. Rev. Biochem., 60:653-88 (1991). When STRs are compared, a distinct spatial pattern of homology is discernable. The transmembrane domains are often the most similar, whereas the N- and C-terminal regions, and the cytoplasmic loop connecting transmembrane segments V and VI are more divergent.

The functional significance of different STR regions has been studied by introducing point mutations (both substitutions and deletions) and by constructing chimeras of different but related STRs. Synthetic peptides corresponding to individual segments have also been tested for activity. Affinity labeling has been used to identify ligand binding sites.

It is conceivable that a foreign receptor which is expressed in yeast will functi nally integrate into the yeast membrane, and there interact with the endogenous yeast G protein. More likely, either the receptor will need to be modified (e.g., by replacing its V-VI loop with that of the yeast STE2 or STE3 receptor), or a compatible G protein should be provided.

If the wild-type exogenous G protein-coupled receptor cannot be made functional in yeast, it may be mutated for this purpose. A comparison would be made of the amino acid sequences of the exogenous receptor and of the yeast receptors, and regions of high and low homology identified. Trial mutations would then be made to distinguish regions involved in ligand or G protein binding, from those necessary for functional integration in the membrane. The exogenous receptor would then be mutated in the latter region to more closely resemble the yeast receptor, until functional integration was achieved. If this were insufficient to achieve functionality, mutations would next be made in the regions involved in G protein binding. Mutations would be made in regions involved in ligand binding only as a last resort, and then an effort would be made to preserve ligand binding by making conservative substitutions whenever possible.

Preferably, the yeast genome is modified so that it is unable to produce the endogenous **a** and α factor receptors in functional form. Otherwise, a positive assay score might reflect the ability of a peptide to activate the endogenous G-coupled receptor, and not the receptor of interest.

G protein

The yeast cell must be able to produce a G-protein which is activated by the exogenous receptor, and which can in turn activate the yeast effector(s). It is possible that the endogenous yeast G protein will be sufficiently homologous to the "cognate" G protein which is natively associated with th exogenous receptor for coupling to occur. More likely, it will be nec ssary to g netically engineer the yeast cell to produce

a foreign Gα subunit which can properly interact with the exogenous receptor. For example, the Gα subunit of the yeast G protein may be replaced by th Gα subunit natively associated with the exogenous receptor (or by a protein substantially homologous with that cognate Gα subunit).

Dietzel and Kurjan, Cell, 50:1001 (1987) demonstrated that rat Gαs functionally coupled to the yeast Gβγ complex. However, rat Gαi2 complemented only when substantially overexpressed, while Gα0 did not complement at all. Kang, et al., Mol. Cell. Biol., 10:2582 (1990). Consequently, with some foreign Gα subunits, it is not feasible to simply replace the yeast Gα.

Preferably, the yeast Gα subunit is replaced by a chimeric Gα subunit in which a portion, e.g., at least about 20, more preferably at least about 40, amino acids, which is substantially homologous with the corresponding residues of the amino terminal of the yeast Gα, is fused to a sequence substantially homologous with the main body of a mammalian (or other exogenous) Gα. While 40 amino acids is the suggested starting point, shorter or longer portions may be tested to determine the minimum length required for coupling to yeast Gβγ and the maximum length compatible with retention of coupling to the exogenous receptor. It is presently believed that only the final 10 or 20 amino acids at the carboxy terminal of the Gα subunit are required for interaction with the receptor.

This chimeric Gα subunit will interact with the exogenous receptor and the yeast Gβγ complex, thereby permitting signal transduction.

Screening and Selection

A marker gene is a gene whose expression causes a phenotypic change which is screenable or selectable. If the change is selectable, the phen typic change creates a difference in the growth or survival rate between cells which express the marker

gene and those which do not. If the change is screenable, the phenotype change creates a difference in some detectable characteristic of the c lls, by which the cells which express the marker may b distinguished from thos which do not. Selection is preferable to screening.

The marker gene must be coupled to the yeast pheromone pathway so that expression of the marker gene is dependent on activation of the G protein. This coupling may be achieved by operably linking the marker gene to a pheromone-responsive promoter. The term "pheromone-responsive promoter" indicates a promoter which is regulated by some product of the yeast pheromone signal transduction pathway, not necessarily pheromone per se. In one embodiment, the promoter is activated by the pheromone pathway, in which case, for selection, the expression of the marker gene should result in a benefit to the cell. A preferred marker gene is the imidazole glycerol phosphate dehydratase gene (HIS3). If a pheromone responsive promoter is operably linked to a beneficial gene, the cells will be useful in screening or selecting for agonists. If it is linked to a deleterious gene, the cells will be useful in screening or selecting for antagonists.

Alternatively, the promoter may be one which is repressed by the pheromone pathway, thereby preventing expression of a product which is deleterious to the cell. With a pheromone-repressed promoter, one screens for agonists by linking the promoter to a deleterious gene, and for antagonists, by linking to it a beneficial gene.

Repression may be achieved by operably linking a pheromone-induced promoter to a gene encoding mRNA which is antisense to at least a portion of the mRNA encoded by the marker gene (whether in the coding or flanking regions), so as to inhibit translation of that mRNA. Repression may also be obtained by linking a pheromone-induced promoter to a gene encoding a DNA-binding r pressor protein, and incorporating a suitable operator

site into the promoter or other suitable region of the marker gene.

Suitable positively selectable (beneficial) genes include the following: *URA3, LYS2, HIS3, LEU2, TRP1; ADE1,2,3,4,5,7,8; ARG1,3,4,5,6,8; HIS1,4,5; ILV1,2,5; THR1,4; TRP2,3,4,5; LEU1,4; MET2,3,4,8,9,14,16,19; URA1,2,4,5,10; HOM3,6; ASP3; CHO1; ARO 2,7; CYS3; OLE1; INO1,2,4; PRO1,3* Countless other genes are potential selective markers. The above are involved in well-characterized biosynthetic pathways.

The imidazoleglycerolphosphate dehydratase (IGP dehydratase) gene (HIS3) is preferred because it is both quite sensitive and can be selected over a broad range of expression levels. In the simplest case, the cell is auxotrophic for histidine (requires histidine for growth) in the absence of activation. Activation leads to synthesis of the enzyme and the cell becomes prototrophic for histidine (does not require histidine). Thus the selection is for growth in the absence of histidine. Since only a few molecules per cell of IGP dehydratase are required for histidine prototrophy, the assay is very sensitive.

In a more complex version of the assay, cells can be selected for resistance to aminothiazole (AT), a drug that inhibits the activity of IGP dehydration. Cells with low, fixed level of expression of HIS3 are sensitive to the drug, while cells with higher levels are resistant. The amount of AT can be selected to inhibit cells with a basal level of HIS3 expression (whatever that level is) but allow growth of cells with an induced level of expression. In this case selection is for growth in the absence of histidine and the presence of a suitable level of AT.

In appropriate assays, so-called counterselectable or negatively selectable genes may be used. Suitable genes include: URA3 (inhibits growth on orotidine-5'-phosphate decarboxylase; growth on 5-fluorotic acid), LYS2 (2-aminoadipate reductase; inhibits growth on α-amino adipate as sole nitrog n source), CYH2

(enc des ribosomal protein L29; cycloh ximide-sensitive allele is dominant to resistant allele), CAN1 (encodes arginine permease; null allele confers r sistance to the arginine analog canavanine), and other recessive drug-resistant markers.

The natural response to induction of the yeast pheromone response pathway is for cells to undergo growth arrest. This is the preferred way to select for antagonists to a ligand/receptor pair that induces the pathway. An autocrine peptide antagonist would inhibit the activation of the pathway; hence, the cell would be able to grow. Thus, the FAR1 gene may be considered an endogenous counterselectable marker. The FAR1 gene is preferably inactivated when screening for agonist activity.

The marker gene may also be a screenable gene. The screened characteristic may be a change in cell morphology, metabolism or other screenable features. Suitable markers include beta-galactosidase (Xgal, C_{12}FDG, Salmon-gal, Magenta-Gal (latter two from Biosynth Ag)), alkaline phosphatase, horseradish peroxidase, exo-glucanase (product of yeast exb1 gene; nonessential, secreted); luciferase; and chloramphericol transferase. Some of the above can be engineered so that they are secreted (although not β-galactosidase). The preferred screenable marker gene is beta-galactosidase; yeast cells expressing the enzyme convert the colorless substrate Xgal into a blue pigment. Again, the promoter may be pheromone-induced or pheromone-inhibited.

Yeast Cells

The yeast may be of any species that possess a G protein-mediated signal transduction pathway and which are cultivatable. Suitable species include Kluyverei lactis, Schizosaccharomyces pombe, and Ustilago maydis; Saccharomyces cerevisiae is preferred. Preferably, it is of a species in which at least some signals are transduced by a pathway in which the G$\beta\gamma$ complex activates the effector. It is suspected that in som species, both Gα-activated and G$\beta\gamma$-activated effectors exist. The term "yeast", as used h rein, includes not only yeast in a strictly

taxonomic sens (i.e., unicellular organisms), but also yeast-like multicellular fungi with pheromone responses mediated by the mating pathway.

The yeast cells of the present invention may be used to test peptides for the ability to interact with an exogenous G protein-coupled receptor. The yeast cells must express both the exogenous G protein-coupled receptor, and a complementary G protein, and these molecules must be presented in such a manner that they can transduce an extracellular signal.

In addition, a gene encoding a selectable or screenable trait must be coupled to the G protein-mediated signal transduction pathway so that the level of expression of the gene is sensitive to the presence or absence of a signal, i.e., binding to the coupled exogenous receptor. This gene may be an unmodified gene already in the pathway, such as the genes responsible for growth arrest. It may be a yeast gene, not normally a part of the pathway, that has been operably linked to a "pheromone-responsive" promoter. Or it may be a heterologous gene that has been so linked. Suitable genes and promoters were discussed above.

It will be understood that to achieve selection or screening, the yeast must have an appropriate phenotype. For example, introducing a pheromone-responsive chimeric HIS3 gene into a yeast that has a wild-type HIS3 gene would frustrate genetic selection. Thus, to achieve nutritional selection, an auxotrophic strain is wanted.

The yeast cells of the present invention optionally possess one or more of the following characteristics:

(a) the endogenous FAR1 gene has been inactivated;

(b) the endogenous SST2 gene, and/or other genes involved in desensitization, has been inactivated;

(c) the endogenous pheromone (a or α factor) recept r gene has b en inactivated; and

(d) the endogenous pheromone genes have been inactivated.

"Inactivation" means that production of a functional gene product is prevented or inhibited. Inactivation may be achieved by deletion of the gene, mutation of the promoter so that expression does not occur, or mutation of the coding sequence so that the gene product is inactive. Inactivation may be partial or total.

Mutants with inactivated supersensitivity-related genes can be identified by conventional genetic screening procedures. The far1 gene was identified as an alpha-factor resistant mutant that remained blue (with fus1-lacZ) on alpha-factor/Xgal. far2, as it turns out, is the same as fus3. Supersensitive mutants could be picked up as constitutive weak blues with fus1-lacZ on Xgal, or as strains that can mate more proficiently with a poor pheromone-secreter.

The DNA sequences of (a) the α and a factor genes, (b) the α and a factor receptors, (c) the FAR1 gene, (d) the SST2 gene, and (e) the FUS1 promoter have been reported in the following references:

MFa1 and MFa2: AJ Brake, C Brenner, R Najarian, P Laybourn, and J Merryweather. Structure of Genes Encoding Precursors of the Yeast Peptide Mating Pheromone a-Factor. In Protein Transport and Secretion. Gething M-J, ed. Cold Spring Harbor Lab, New York, 1985.

MFα1 and MFα2: Singh, A. EY Chen, JM Lugovoy, CN Chang, RA Hitzeman et al. 1983. Saccharomyces cerevisiae contains two discrete genes coding for the α-pheromone. Nucleic Acids Res. 11:4049; J Kurjan and I Herskowitz. 1982. Structure of a yeast pheromone gene (MF): A putative α-factor precursor contains four tandem copies of mature α-fact r. Cell 30:933.

STE2 and STE3: AC Burkholder and LH Hartwell. 1985. The yeast α-factor rec pt r: Structural prop rties deduced from the sequence of the *STE2* gene. Nucleic Acids Res. 13:8463; N Nakayama, A Miyajima, and K Arai. 1985. Nucleotide sequences of *STE2* and *STE3*, cell type-specific sterile genes from *Saccharomyces cerevisiae*. EMBO J. 4:2643; DC Hagen, G McCaffrey, and GF Sprague, Jr. 1986. Evidence the yeast *STE3* gene encodes a receptor for the peptide pheromone a factor: Gene sequence and implications for the structure of the presumed receptor. Proc Natl Acad Sci 83:1418.

FAR1: F Chang and I Herskowitz. 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. Cell 63:999.

SST2: C Dietzel and J Kurjan. 1987. Pheromonal regulation and sequence of the *Saccharomyceres cerevisiae SST2* gene: A model for desensitization to pheromone. Mol Cell Biol 7: 4169.

FUS1: J Trueheart, JD Boeke, and GR Fink. 1987. Two genes required for cell fusion during yeast conjugation: Evidence for a pheromone-induced surface protein. Mol Cell Biol 7:2316.

The various essential and optional features may be imparted to yeast cells by one or more of the following means: isolation of spontaneous mutants with one or more of the desired features; mutation of yeast by chemical or radiation treatment, followed by selection; and genetic engineering of yeast cells to introduce, modify or delete genes.

Peptide

The term "peptide" is used herein to refer to a chain of two or more amino acids, with adjacent amino acids joined by peptide (-NHCO-) bonds. Thus, the peptides of the present invention includ oligopeptides, polypeptides, and proteins. Pr ferably, the peptides of the present invention are 2 to 200, more preferably 5 to 50, amino acids in length. The minimum peptide

length is chiefly dictated by the need to obtain sufficient potency as an agonist or antagonist. Th maximum peptide length is only a function of synthetic convenience once an active peptide is identified.

For initial studies, a 13-amino acid peptide was especially preferred as that is the length of the mature yeast α factor.

Peptide Libraries

A "peptide library" is a collection of peptides of many different sequences (typically more than 1000 different sequences), which are prepared essentially simultaneously, in such a way that, if tested simultaneously for some activity, it is possible to characterize the "positive" peptides.

The peptide library of the present invention takes the form of a yeast cell culture, in which essentially each cell expresses one, and usually only one, peptide of the library. While the diversity of the library is maximized if each cell produces a peptide of a different sequence, it is usually prudent to construct the library so there is some redundancy.

In the present invention, the peptides of the library are encoded by a mixture of DNA molecules of different sequence. Each peptide-encoding DNA molecule is ligated with a vector DNA molecule and the resulting recombinant DNA molecule is introduced into a yeast cell. Since it is a matter of chance which peptide-encoding DNA molecule is introduced into a particular cell, it is not predictable which peptide that cell will produce. However, based on a knowledge of the manner in which the mixture was prepared, one may make certain statistical predictions about the mixture of peptides in the peptide library.

It is convenient to speak of the peptides of the library as being composed of constant and variable residues. If the nth residue is th sam for all peptides of the library, it is said to b constant. If the nth residue vari s, depending on the

p ptide in question, the reside is a variable one. The peptides of the library will have at least ne, and usually more than one, variable residue. A variable residue may vary among any of two to all twenty of th genetically encoded amino acids; the variable residues of the peptide may vary in the same or different manner. Moreover, the frequency of occurrence of the allowed amino acids at a particular residue position may be the same or different. The peptide may also have one or more constant residues.

There are two principal ways in which to prepare the required DNA mixture. In one method, the DNAs are synthesized a base at a time. When variation is desired, at a base position dictated by the Genetic Code, a suitable mixture of nucleotides is reacted with the nascent DNA, rather than the pure nucleotide reagent of conventional polynucleotide synthesis.

The second method provides more exact control over the amino acid variation. First, trinucleotide reagents are prepared, each trinucleotide being a codon of one (and only one) of the amino acids to be featured in the peptide library. When a particular variable residue is to be synthesized, a mixture is made of the appropriate trinucleotides and reacted with the nascent DNA.

Once the necessary "degenerate" DNA is complete, it must be joined with the DNA sequences necessary to assure the expression of the peptide, as discussed in more detail below, and the complete DNA construct must be introduced into the yeast cell.

Expression

The expression of a peptide-encoding gene in a yeast cell requires a promoter which is functional in yeast. Suitable promoters include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255, 2073 (1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7, 149 (1968); and Holland et al. Biochemistry 17, 4900 (1978)), such as en las , glyceraldehyde-3-phosphat

dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinas , glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, trios phosphate isomerase, ph sphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization. Finally, promoters that are active in only one of the two haploid mating types may be appropriate in certain circumstances. Among these haploid-specific promoters, the pheromone promoters MFa1 and MFα1 are of particular interest.

In constructing suitable expression plasmids, the termination sequences associated with these genes may also be ligated into the expression vector 3' of the heterologous coding sequences to provide polyadenylation and termination of the mRNA.

Vectors

The vector must be capable of replication in a yeast cell. It may be a DNA which is integrated into the host genome, and thereafter is replicated as a part of the chromosomal DNA, or it may be DNA which replicates autonomously, as in the case of a plasmid. In the latter case, the vector must include an origin of replication which is functional in the host. In the case of an integrating vector, the vector may include sequences which facilitate integration, e.g., sequences homologous to host sequences, or encoding integrases.

Besides being capable of replication of yeast cells, it is convenient if the vector can also be replicated in bacterial cells, as many g netic manipulations are more conveniently carried out therein. Shuttle vect rs capable of replication in

both yeast and bacterial cells include YEps, YIps, and the pRS series.

Periplasmic Secretion

The cytoplasm of the yeast cell is bounded by a lipid bilayer called the plasma membrane. Between this plasma membrane and the cell wall is the periplasmic space. Peptides secreted by yeast cells cross the plasma membrane through a variety of mechanisms and thereby enter the periplasmic space. The secreted peptides are then free to interact with other molecules that are present in the periplasm or displayed on the outer surface of the plasma membrane. The peptides then either undergo re-uptake into the cell, diffuse through the cell wall into the medium, or become degraded with the periplasmic space.

The peptide library may be secreted into the periplasm by one of two distinct mechanisms, depending on the nature of the expression system to which they are linked. In one system, the peptide may be structurally linked to a yeast signal sequence, such as that present in the α-factor precursor, which directs secretion through the endoplasmic reticulum and Golgi apparatus. Since this is the same route that the receptor protein follows in its journey to the plasma membrane, opportunity exists in cells expressing both the receptor and the peptide library for a specific peptide to interact with the receptor during transit through the secretory pathway. This has been postulated to occur in mammalian cells exhibiting autocrine activation. Such interaction would likely yield activation of the linked pheromone response pathway during transit, which would still allow identification of those cells expressing a peptide agonist. For situations in which peptide antagonists to externally applied receptor agonist are sought, this system would still be effective, since both the peptide antagonist and receptor would be delivered to the outside of the cell in concert. Thus, those cells producing an antagonist would be selectable, since the

peptid agonist w uld properly and timely situated to prevent the receptor from being stimulated by the externally applied agonist.

An alternative mechanism for delivering peptides to the periplasmic space is to use the ATP-dependent transporters of the STE6/MDR1 class. This class of transport pathway and the signals that direct a protein or peptide to this pathway are not as well characterized as that of the endoplasmic reticulum based secretory pathway. Nonetheless, these transporters apparently can efficiently export certain peptides directly across the plasma membrane, without the peptides having to transit the ER/Golgi pathway. We anticipate that at least a subset of peptides can be secreted through this pathway by expressing the library in context of the a-factor prosequence and terminal tetrapeptide. The possible advantage of this system is that the receptor and peptide do not come into contact until both are delivered to the external surface of the cell. Thus, this system strictly mimics the situation of an agonist or antagonist that is normally delivered from outside the cell. Use of either of the described pathways is within the scope of the invention.

The present invention does not require periplasmic secretion, or, if such secretion is provided, any particular secretion signal or transport pathway.

"Substantial Homology"

The term "substantially homologous", when used in connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence, giving rise to a homology in conformation and thus to similar biological activity. The term is not intended to imply a common evolution of the sequences.

Typically, "substantially homologous" sequences are at least 50%, more preferably at least 80%, identical in sequence, at least over any regions known to b involved in the desired activity. Most preferably, no more than fiv residues, other

than at the termini, are different. Preferably, the divergence in sequence, at least in the aforementioned regions, is in the form of "conservative modifications".

"Conservative modifications" are defined as

(a) conservative substitutions of amino acids as hereafter defined; and

(b) single or multiple insertions or deletions of amino acids at the termini, at interdomain boundaries, in loops or in other segments of relatively high mobility. Preferably, except at the termini, no more than about five amino acids are inserted or deleted at a particular locus, and the modifications are outside regions known to contain binding sites important to activity.

Conservative substitutions are herein defined as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:
Ala, Ser, Thr (Pro, Gly)

II. Polar, negatively charged residues: and their amides
Asp, Asn, Glu, Gln

III. Polar, positively charged residues:
His, Arg, Lys

IV. Large, aliphatic, nonpolar residues:
Met, Leu, Ile, Val (Cys)

V. Large, aromatic residues:
Phe, Tyr, Trp

Residues Pro, Gly and Cys are parenthesized because they have special conformational roles. Cys participates in formation of disulfide bonds. Gly imparts flexibility to the chain. Pro imparts rigidity to the chain and disrupts alpha helices. These residues may be essential in certain regions of the polypeptide, but substitutable elsewhere.

Two regulatory DNA sequences (e.g., promoters) are "substantially homologous" if th y have substantially the same regulatory effect as a result of a substantial identity in nucleotide sequence. Typically, "substantially homologous" sequences are at least 50%, more preferably at least 80%, identical, at least in regions known to be involved in the desired regulation. Most preferably, no more than five bases are different.

Example 1

In this example, we describe a pilot experiment in which haploid cells were engineered to be responsive to their own pheromones. (Note that in the examples, functional genes are capitalized and inactivated genes are in lower case.) For this purpose we constructed recombinant DNA molecules designed to:

i. place the coding region of *STE2* under the transcriptional control of elements which normally direct the transcription of *STE3*. This is done in a plasmid that allows the replacement of genomic *STE3* of *S.cerevisiae* with sequences wherein the coding sequence of *STE2* is driven by *STE3* transcriptional control elements.

ii. place the coding region of *STE3* under the transcriptional control of elements which normally direct the transcription of *STE2*. This is done in a plasmid which will allow the replacement of genomic *STE2* of *S.cerevisiae* with sequences wherein the coding sequence of *STE3 is* driven by *STE2* transcriptional control elements.

STE2 was made available as a 4.3 kb BamHI fragment cloned into YEp24 (Botstein et al, Gene 8: 117, 1979). The 4.3 kb BamHI fragment was subcloned into pALTER (Protocols and Applications Guide, 1991, Promega Corporation, Madison, WI), and Spe I sites were introduced 7 nucleotides upstream f the start codon and 9

nucleotides downstream of the stop codon with the following respective mutagenic oligonucleotides:

5'GTTAAGAACCATATACTAGTATCAAAAATGTCTG and

5'TGATCAAAATTTACTAGTTTGAAAAAGTAATTTCG.

(The start and stop codons are in bold type and the Spe I sites are underlined). The mutated *STE2* was moved as a BamHI fragment into the Bam HI site of YIp19 (J. Broach, unpublished) to generate YIp-STE2. *STE3* was made available by Dr. J. Broach as a 3.1 kb fragment cloned into pBLUESCRIPT-KS II (Stratagene, 11011 North Torrey Pines Road, La Jolla, CA 92037). The plasmid contains two Spe I sites: one in the polylinker of pBLUESCRIPT-KS II and one near the 5' end of the *STE3*-containing fragment. These were eliminated by digesting with Spe I, filling in the overhangs, and recircularizing by blunt-end ligation. The sites were successfully introduced using T7-GEN protocol of United States Biochemical (T7-GEN *In Vitro* Mutagenesis Kit, Descriptions and Protocols, 1991, United States Biochemical, P.O. Box 22400, Cleveland, Ohio 44122) for single-stranded mutagenesis using the mutagenic oligonucleotides

5' GGCAAAATACTAGTAAAATTTTCATGTC and

5' GGCCCTTAACACACTAGTGTCGCATTATATTTAC.

(The start and stop codons are in bold type and the Spe I sites are underlined.) The mutated *STE3* was subcloned as a 2.8 kb Xba I-to-Kpn I fragment into a derivative of pRS406 (Sikorski and Hieter, Genetics 122:19-27, 1989), made by elimination of its unique Spe I site, to generate pRS-STE3.

The coding regions of *STE2* and *STE3* were exchanged by digesting both YIp-STE2 and pRS-STE3 with Spe I, isolating the inserts and the vectors, and cloning the coding region of *STE2* into the pRS vector and the coding region of *STE3* into the YIp vector. The resulting plasmids, pRS-STE2 and YIp-STE3 contain, respectively, the STE2 coding region transcribed by the STE3 promoter (ste3::STE2::ste3) and the STE3 coding region transcribed by the STE2 promoter (ste2::STE3::ste2). Both plasmids contain the selectable marker *URA3*.

Haploid yeast of mating type a which had been engineered to express HIS3 under th control of the pheromone-inducible FUS1 promoter were transformed with YIp-STE3, and transformants expressing URA3 were s lect d. Thes transformants, which express both Ste2p and Ste3p, were plated on 5-fluoroorotic acid to allow the selection of clones which had lost the endogenous *STE2*, leaving in its place the heterologous, integrated *STE3*. Such cells exhibited the ability to grow on media deficient in histidine, indicating autocrine stimulation of the pheromone response pathway.

Similarly, haploids of mating type α that can express HIS3 under the control of the pheromone-inducible FUS1 promoter were transformed with pRS-STE2 and selected for replacement of their endogenous STE3 with the integrated STE2. Such cells showed, by their ability to grow on histidine-deficient media, autocrine stimulation of the pheromone response pathway.

Example 2

In this example, yeast strains are constructed which will facilitate selection of clones which exhibit autocrine activation of the pheromone response pathway. To construct appropriate yeast strains, we will use: the YIp-STE3 and pRS-STE2 knockout plasmids described above, plasmids available for the knockout of FAR1, SST2, and HIS3, and mutant strains that are commonly available in the research community. The following haploid strains will be constructed, using one-step or two-step knockout protocols described in Meth. Enzymol 194:281-301, 1991:

1. *MATα ste3::STE2::ste3 far1 sst2 FUS1::HIS3*
2. *MATa ste2::STE3::ste2 far1 sst2 FUS1::HIS3*
3. *MATα ste3::STE2::ste3 far1 sst2 mfα1 mfα2 FUS1::HIS3*
4. *MATa ste2::STE3::ste2 far1 sst2 mfa1 mfa2 FUS1::HIS3*

Strains 1 and 2 will be tested for their ability to grow on histidine-deficient media as a result of autocrine stimulation of their pheromone response pathways by the pheromones which they

secr te. If th se tests prove successful, strain 1 will be modified to inactivat endog nous *MFα1* and *MFα2*. The resulting strain 3, *MATα far1 sst2 ste3::STE2::ste3 FUS1::HIS3 mfa1 mfa2*, should no long r display the s l ctabl phenotype (i.e., the stain should be auxotrophic for histidine). Similarly, strain 2 will be modified to inactivate endogenous *MFa1* and *MFa2* . The resulting strain 4, *MATa far1 sst2 ste2::STE3::ste2 FUS1::HIS3 mfa1 mfa2*, should be auxotrophic for histidine.

Example 3

In this example, a synthetic oligonucleotide encoding a pheromone is expressed so that the peptide is secreted into the periplasm.

i. The region of *MFα1* which encodes mature α-factor has been replaced via single stranded mutagenesis with restriction sites that can accept oligonucleotides with Afl II and Bgl II ends. Insertion of oligonucleotides with Afl II and Bgl II ends will yield plasmids which encode proteins containing the MFα1 signal and leader sequences upstream of the sequence encoded by the oligonucleotides. The MFα1 signal and leader sequences should direct the processing of these precursor proteins through the pathway normally used for the secretion of mature a-factor.

MFα1, available as a 1.8 kb EcoRI insert in pDA6300 (available from J. Thorner, unpublished), was cloned as an EcoRI fragment into Promega's pALTER (see Figure 2) for the introduction by oligonucleotide-directed mutagenesis of two mutations, performed simultaneously on the minus strand of *MFα1* using Promega's Altered Sites protocol. One mutation involved the introduction of a Hind III site upstream of the ATG of *MFα1* using the mutagenic oligonucleotide

5' CATACACAATATAAAGCTTTAAAAGAATGAG.

(The newly created Hind III site is underlined and the start codon of MFα1 is in bold type). The other mutation involves removing the coding region of the mature α-factor while introducing sites for the later insertion of oligonucleotides with AfL II and Bcl I termini. (The Bc1 I acceptor site will not

b Bcl I, since it can b *dam* methylated, but rather Bgl II which will not be *dam* methylated. Bcl I and Bgl II share compatible overhangs). Th mutag nic oligonucl otid to accomplish these latter modifications is

5'CTAAAGAAGAAGGGGTATCTTTGCTTAAGCTCGAGATCTCGACTGATAACAACAGTGTAG

The result of the two mutations is the creation in pALTER of an MFα1 translation module which contains a Hind III site 7 nucleotides upstream of the MFα1 initiation codon, an Afl II site at the positions which encode the KEX2 processing site in the leader peptide, and Xho I and Bgl II sites in place of all sequences from the leader-encoding sequences up to and including the MF1 stop codon. The CTCG sequence are spacer sequences which should allow efficient double digestion with Afl II and Bgl II while allowing the possibility of cutting between the Afl II and Bgl II sites with Xho I.

The MFα1 translation module was moved from pALTER to a high-copy plasmid which contains the ADC promoter and which is constructed as follows (see Figure 3): the ADC1 promoter and 5' flanking sequences were removed from pAAH5 (Meth Enzymol 101:192, 1983) with BamHI and Hind III, and the 1.5 kb fragment is cloned into the Bam HI and Hind III sites of the polylinker of pR426 (Christianson et al., Gene 110:119-122, 1992) yielding pRS-ADC. pRS-ADC has a unique Hind III site into which the MFα1 translation module can be inserted as a Hind III fragment. Oligonucleotides cloned into the Afl II and Bgl II sites of the resulting expression plasmid, designated pADC-MFα1, will be expressed under the transcriptional control of the ADC promoter and in the context of MFα1 signal and leader peptides.

ii. The region of *MFa1* which encodes mature a-factor will be replaced via single stranded mutagenesis with restriction sites that can accept oligonucleotides with Xho I and Afl II ends. Insertion of oligonucleotides with Xho I and Afl II ends will yield plasmids which encode prot ins containing the MFa1 leader sequences upstream of the sequence encoded by the

oligonucleotides. The MFa1 leader sequences should direct the processing of these precursor proteins through the pathway normally used for the secretion of matur a-factor.

MFa1, available as a 1.6 kb EcoRI insert in pKK1 (Kuchler et al EMBO J. 8: 3973-84, 1989) was cloned as an EcoRI fragment into pALTER (Figure 5) for oligonucleotide-directed mutagenesis of the minus strand of MFa1 using Promega's Altered Sites protocol. One mutation involved the introduction of a Hind III site upstream of the ATG of MFa1 using the mutagenic oligonucleotide
5' CCAAAATAAGTACAAAGCTTTCGAATAGAAATGCAACCATC.
(The newly created Hind III site is underlined and start codon is in bold type). The other mutation involves removing the coding region of the mature a-factor while introducing Xho I and Afl II site for the later insertion of oligonucleotides with Xho I and Afl II termini. The mutagenic oligonucleotide to accomplish these latter modifications is
5' GCCGCTCCAAAAGAAAAGACCTCGAGCTCGCTTAAGTTCTGCGTACAAAAACGTTGTTC,
where The Xho I And Afl II sites are underlined and the stop codon is in bold type. The result of the two mutations is the creation in pALTER of an MFa1 translation module which contains a Hind III site 10 nucleotides upstream of the MFa1 initiation codon and a linker in place of sequences encoding mature a-factor. The CTCG sequence is a spacer sequence which should allow efficient double digestion with Xho I and Afl II while allowing the possibility of cutting between the Xho I and Afl II site with Sac I. The MFa translation module will be moved from pALTER to a high-copy plasmid which contains the ADC promoter as described above for the MF1 translation module. Oligonucleotides cloned into the Xho I and Afl II sites of the resulting expression plasmid, designated pADC-MFa, will be expressed under the transcriptional control of the ADC promoter and in the context of MFa1 leader peptides (Figure 6).

iii. An oligonucleotide encoding mature alpha-factor will be cloned into the MFα translation m dule of pADC-MFα, and haploid yeast of mating type whose ndogenous alpha-factor-encoding

genes have been mutated to liminate endogenous production of alpha-factor (strain 3 in Example 2 above) will be transformed with the alpha-factor- xpressing plasmid. Transformants will be screened for their capacity to produce alpha-factor. Similarly, an oligonucleotide encoding the a-factor peptid will be clon d into the MFa translation module of pADC-MFa, and a-cells deficient in production of endogenous a-factor (strain 4 in Example 2 above below) will be transformed with the resulting plasmid. Transformants will be screened for their capacity to produce a-factor.

This example will demonstrate the ability to engineer yeast such that they secrete oligonucleotide-encoded peptides (in this case their pheromones) through the pathway normally used for the secretion of their pheromones.

Example 4

This example will demonstrate the utility of the autocrine system for the discovery of peptides which behave as functional pheromone analogues.

If these tests are successful, Strain 3 (see Example 2 above) will be transformed with pADC-MFα containing oligonucleotides encoding random tridecapeptides for the isolation of functional α-factor analogues (Figure 4). Strain 4 (see Example 2 above) will be transformed with pADC-MFa containing oligos of random sequence for the isolation of functional a-factor analogues (Figure 6). Colonies of either strain which can grow on histidine-deficient media following transformation will be expanded for the preparation of plasmid DNA, and the oligonucleotide cloned into the expression plasmid will be sequenced to determine the amino acid sequence of the peptide which presumably activates the pheromone receptor. This plasmid will then be transfected into an isogenic strain to confirm its ability to encode a peptide which activates the pheromone receptor. Successful completion of these experiments will demonstrate the potential of the system for the discovery of

peptides which can activate membrane rec ptors coupled to the pheromone respons pathway.

Random oligonucleotides to be expressed by the expression plasmid pADC-MFα will encode tridecapeptides constructed as

5' CGTGAAGCTTAAGCGTGAGGCAGAAGCT(NNK)$_{13}$TGATCATCCG,

where N is any nucleotide, K is either T or G at a ratio of 40:60 (see Proc Natl Aced Sci 87:6378, 1990; *ibid* 89:5393, 1992), and the Afl II and Bcl I sites are underlined. This oligonucleotide is designed such that: the Afl II and Bcl I sites permit inserting the oligos into the Afl II and Bgl II site of pADC-MFα1 (see Figure 4); the Hind II site just 5' to the Afl II site in the 5' end of the oligo allows future flexibility with cloning of the oligos; the virtual repeat of GAGGCT and the GAGA repeats which are present in the wild-type sequence and which can form triple helixes are changed without changing the encoded amino acids. The random oligonucleotides described above will actually be constructed from the following two oligos:

5' CGTGAAGCTTAAGCGTGAGGCAGAAGCT and

5' CGGATGATCA(ZNN)$_{13}$AGCTTCTG,

where Z is either A or C at a ratio of 40:60. The oligos will be annealed with one another and repetitively filled in, denatured, and reannealed (Kay et at, Gene, in press). The double-stranded product will be cut with Afl II and Bcl I and ligated into the Afl II-and Bgl II-digested pADC-MFα. The Bgl II/Bcl I joint will create a TGA stop codon for termination of translation of the randomers (Figure 4). Because of the TA content of the Afl overhang, the oligos will be ligated to the Aft II-and Bgl II-digested pADC-MFα at 4°C.

Random oligonucleotides to be expressed by the expression plasmid pADC-MFa will encode monodecapeptides constructed as

5' GGTACTCGAGTGAAAAGAAGGACAAC(NNK)$_{11}$TGTGTTATTGCTTAAGTACG,

where N is any nucleotide, K is either T or G at a ratio of 40:60 (see Proc Natl Acad set 87:6378, 1990; *ibid* 89:5393, 1992), and the Xho I and Afl II sites are underlined. When cloned int the Xho I and Afl II sites of pADC-MFa, the propeptides expressed under the control of the ADC promoter will contain th entire

leader peptid of MFa1, follow d by 11 random amino acids, followed by triplets ncoding CVIA (the C-terminal tetrapeptide of wild-type a-factor). Processing of the propeptide should result in the secretion of dodecapeptides which contain 11 random amino acids followed by a C-terminal, farnesylated, carboxymethylated cysteine.

Using the procedure described above, the oligonucleotides for expression in pADC-MFa will actually be constructed from the following two oligos:

5' GGTACTCGAGTGAAAAGAAGGACAAC and

5' CGTACTTAAGCAATAACAca(ZNN)$_{11}$GTTGTCC,

where Z is either A or C at a ratio of 40:60, and the Xho I and Afl II sites are underlined.

TABLE I: HUMAN G PROTEIN-COUPLED SEVEN TRANSMEMBRANE RECEPTORS: REFERENCES FOR CLONING

Rec ptor	Reference
α_{1A}-adrenergic receptor	Bruno et al. (1991)
α_{1B}-adrenergic receptor	Ramarao et al. (1992)
α_2-adrenergic receptor	Lomasney et al. (1990)
α_{2B}-adrenergic receptor	Weinshank et al. (1990)
β_1-adrenergic receptor	Frielle et al. (1987)
β_2-adrenergic receptor	Kobilka et al. (1987)
β_3-adrenergic receptor	Regan et al. (1988)
m_1 AChR, m2 AChR, m_3 AChR, m_4 AChR	Bonner et al. (1987) Peralta et al. (1987)
m5 AChR	Bonner et al. (1988)
D_1 dopamine	Dearry et al. (1990) Zhou et al. (1990) Sunahara et al. (1990) Weinshank et al. (1991)
D_2 dopamine	Grandy et al. (1989)
D_3 dopamine	Sokoloff et al. (1990)
D_4 dopamine	Van Tol et al. (1991)
D_5 dopamine	M. Caron (unpub.) Weinshank et al. (1991)
A1 adenosine	Libert et al. (1992)
adenosine A2b	Pierce et al. (1992)
5-HT1a	Kobilka et al. (1987) Fargin et al. (1988)
5-HT1b	Hamblin et al. (1992) Mochizuki et al. (1992)

5HT1-like	L vy et al. (1992a)
5-HT1d	Levy et al. (1992b)
5HT1d-like	Hamblin and Metcalf (1991)
5HT1d beta	Demchyshyn et al. (1992)
substance K (neurokinin A)	Gerard et al. (1990)
substance P (NK1)	Gerard, et al. (1991); Takeda et al. (1991)
f-Met>Leu-Phe	Boulay et al. (1990) Murphy & McDermott (1991) DeNardin et al. (1992)
angiotensin II type 1	Furuta et al. (1992)
mas proto-oncogene	Young et al. (1986)
endothelin ETA	Hayzer et al. (1992) Hosoda et al. (1991)
endothelin ETB	Nakamuta et al. (1991) Ogawa et al. (1991)
thrombin	Vu et al. (1991)
growth hormone-releasing hormone (GHRH)	Mayo (1992)

Receptor	Reference
vasoactive intestinal peptide (VIP)	Sreedharan et al. (1991)
oxytocin	Kimura et al., (1992)

somatostatin SSTR1 and SSTR2 SSTR3	Yamada et al. (1992a) Yamada et al. (1992b)
cannabinoid	Gerard et al. (1991)
follicle stimulating hormone (FSH)	Minegish et al. (1991)
LH/CG	Minegish et al. (1990)
thyroid stimulating hormone (TSH)	Nagayama et al. (1989) Libert et al. (1989) Misrahi et al. (1990)
thromboxane A2	Hirata et al. (1991)
platelet-activating facor (PAF)	Kunz et al. (1992)
C5a anaphylatoxin	Boulay et al. (1991) Gerard and Gerard (1991)
Interleukin 8 (IL-8) IL-8RA	Holmes et al. (1991)
IL-8RB	Murphy and Tiffany (1991)
Delta Opioid	Evans et al. (1992)
Kappa Opioid	Xie et al. (1992)
mip-1/RANTES	Neote et al. (1993) Murphy et al., in press
Rhodopsin	Nathans and Hogness (1984)
Red opsin, Green opsin, Blue opsin	Nathans, et al. (1986)
metabotropic glutamat mGluR1-6	Tanabe et al. (1992)

histamin H2	Gantz t al. (1991)
ATP	Julius, David (unpub.)
neuropeptide Y	Herzog et al. (1992) Larhammar et al. (1992)
amyloid protein precursor	Kang et al. (1987) Mita, et al. (1988) Lemaire et al. (1989)
insulin-like growth factor II	Kiess et al. (1988)
bradykinin	Hess et al. (1992) Merck
gonadotropin-releasing hormone	Chi et al. (1993)
cholecystokinin	Pisegna et al. (1992)
melanocyte stimulating hormone receptor	Chhajlane et al. (1992) Mountjoy et al. (1992)
antidiuretic hormone receptor	Birnbaumer et al. (1992)
glucagon receptor	Sprecher et al. (1993) unpub. ZymoGenetics
adrenocorticotropic hormone II	Mountjoy et al. (1992)

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CLAIMS

1. A yeast cell which expresses a heterologous G-protein coupled receptor, a G-protein capable of transducing a signal from said receptor to genes in the pheromone signal pathway of said cell, and a heterologous peptide, where, if said peptide is an agonist or antagonist for said receptor, it will stimulate or inhibit, respectively, said pheromone signal pathway, and where said stimulation or inhibition is a screenable or selectable event.

2. The yeast cell of claim 1 wherein the endogenous pheromone receptor is not functionally expressed.

3. The yeast cell of claim 1 wherein the peptide is secreted by the cell into the periplasmic space, from which it interacts with said receptor.

4. The yeast cell of claim 3, wherein the peptide is expressed in the form of a precursor peptide comprising a cleavable leader peptide and a mature peptide, and the leader peptide is substantially homologous to the leader peptide of the wild-type pheromone of said cell.

5. The yeast cell of claim 4 wherein the wild-type leader peptide is that of the Saccharomyces cerevisiae α factor or **a** factor.

6. The yeast cell of claim 4 in which the wild-type pheromone is not secreted.

7. The yeast cell of claim 3 wherein the peptide is also expressed in a nonsecretory form.

8. The yeast cell of claim 1 wherein the cell is a mutant strain having a reduced propensity, relative to the wild-type strain, to have its pheromone signal pathway desensitized through repeated or prolonged stimulation thereof.

9. The yeast cell of claim 8 in which the SST2 gene is not functionally expressed.

10. The yeast cell of claim 1, in which the FAR1 gene is not functionally expressed.

11. The yeast cell of claim 1, further comprising a selectable marker that is activated by the pheromone signal pathway.

12. The yeast cell of claim 11, said selectable marker comprising a pheromone-responsive promoter which is substantially homologous with an endogenous pheromone-responsive promoter, operably linked to a foreign sel ctable gene.

13. The yeast cell of claim 12 wherein the selectable gene is an IGP dehydratase gene.

14. The yeast cell of claim 12 wherein the homologous wild-type promoter is the FAR1 promoter.

15. The yeast cell of claim 1 wherein the cells belong to the species Saccharomyces cerevisiae.

16. A yeast culture comprising a plurality of yeast cells according to claim 1, said yeast cells collectively expressing a peptide library.

17. A method of assaying a peptide for agonist or antagonist activity against a non-yeast receptor which comprises providing yeast cells according to claim 1, which cells functionally express said receptor and said peptide, and determining whether the pheromone signal pathway is activated or inhibited by said peptide.

18. The method of claim 17 in which the cells comprise a pheromone-responsive selectable marker, and cells are selected for expression of a peptide having the desired agonist or antagonist activity.

19. The method of claim 17 in which the cells comprise a pheromone-responsive screenable marker, and cells are screened for expression of a peptide having the desired agonist or antagonist activity.

20. A method of assaying a peptide library for agonist or antagonist activity against a non-yeast receptor which comprises providing a yeast culture according to claim 16, which cells each functionally express said receptor and a peptide of said library, said culture collectively expressing the entire peptide library, and determining whether the pheromone signal pathway is activated or inhibited by said peptides in each of the cells of said culture.

21. A method of assaying a peptide for agonist or antagonist activity against a non-yeast receptor which comprises providing yeast cells according to claim 20, which cells functionally express said receptor, and said peptide, and determining whether the pheromone signal pathway is activated or inhibited by said peptide.

22. The method of claim 21 in which the cells comprise a pheromone-responsive selectable mark r, and cells are selected for expression of a peptide having the desired agonist or antagonist activity.

23. The method of claim 22 in which the cells comprise a pheromone-responsive screenable marker, and cells are screened for expression of a peptide having the desired agonist or antagonist activity.

24. The yeast cell of claim 1 in which the peptide is an agonist.

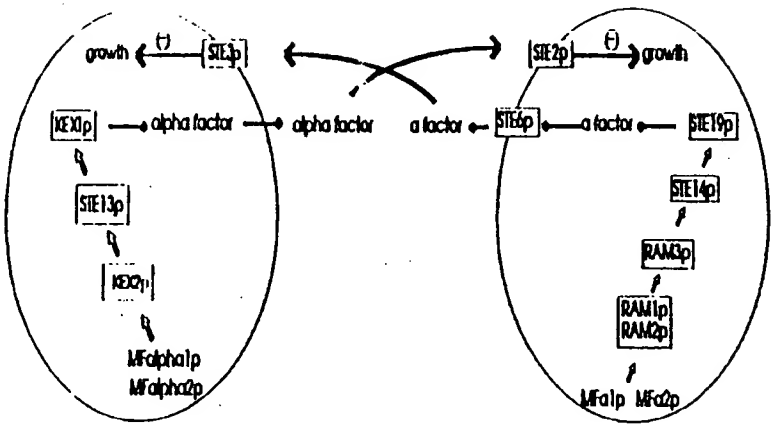
25. The yeast cell of claim 1 in which the peptide is an antagonist.

26. The yeast cell of claim 9 in which the FAR1 gene is not functionally expressed.

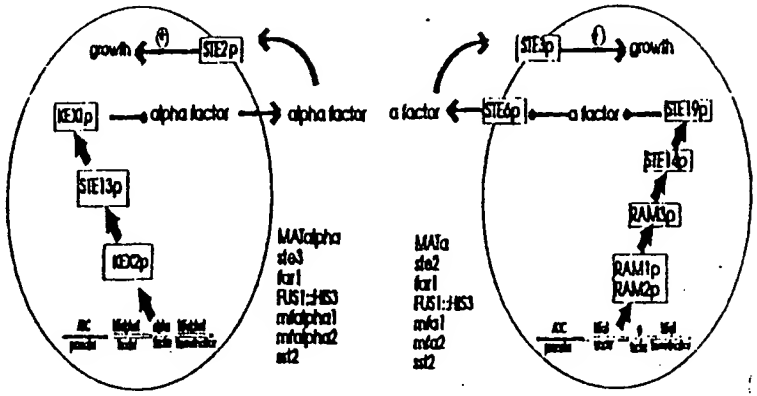
27. The yeast cell of claim 1 wherein the Gα subunit of the G protein is chimeric.

28. The yeast cell of claim 27 wherein the amino terminal portion of the Gα subunit is substantially homologous with the Gα subunit of a yeast G protein and the remainder is substantially homologous with the corresponding portion of a Gα subunit of a heterologous G protein.

Synthesis, Release, and Targets of Mating Pheromones



Stage 2



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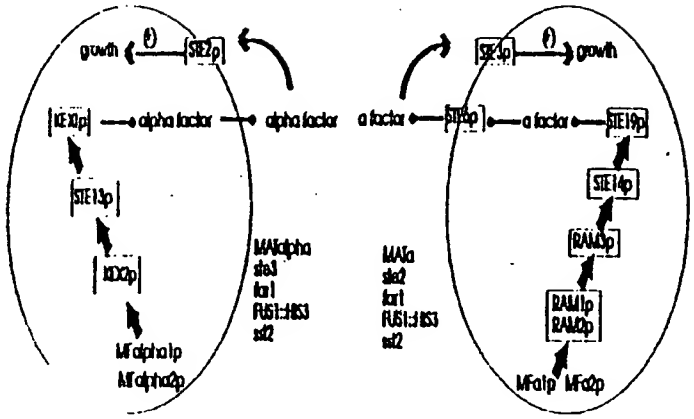

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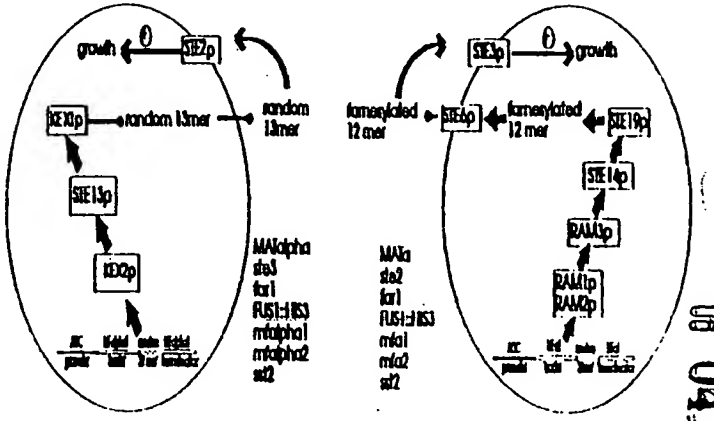
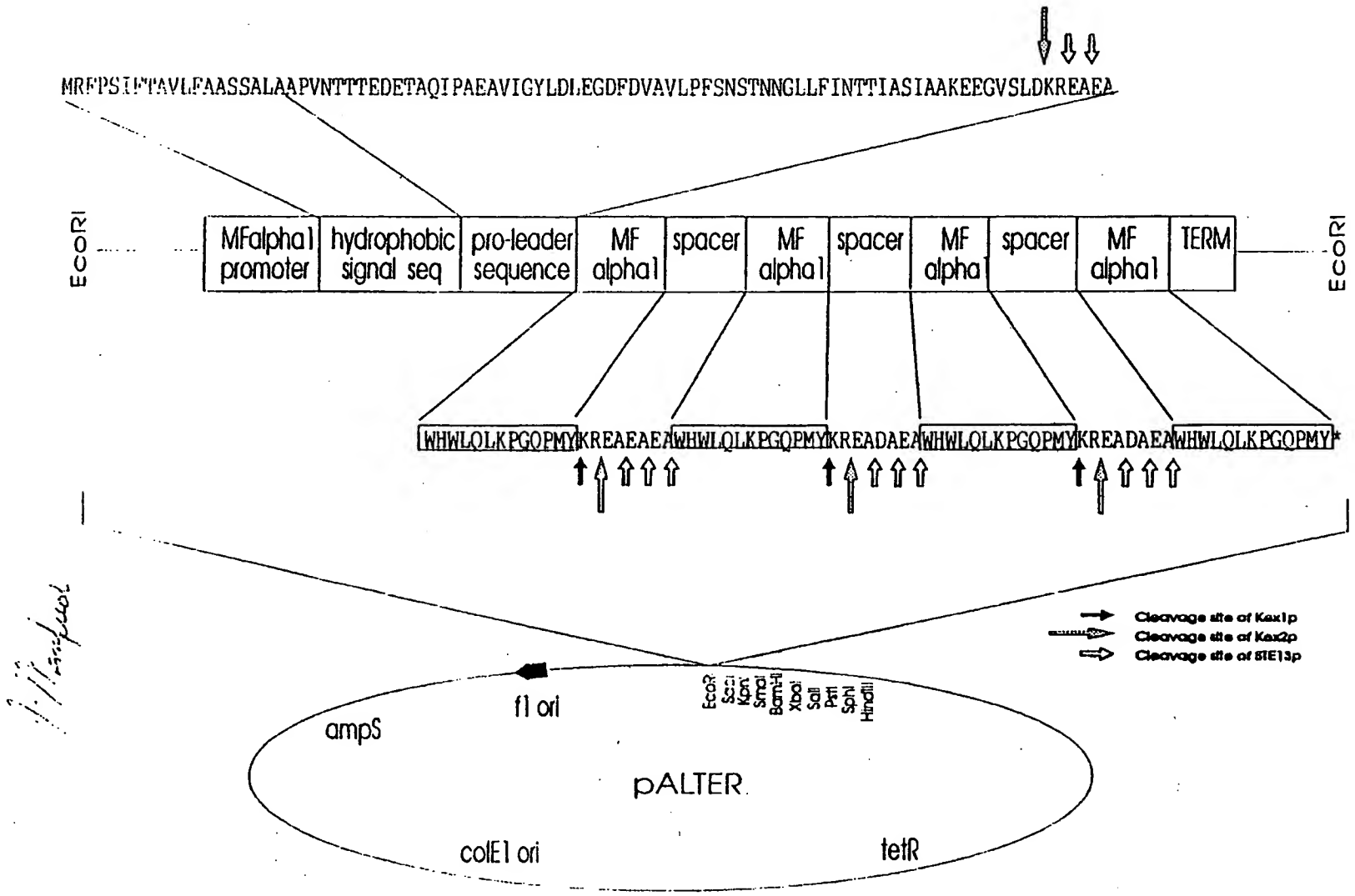


Figure 1



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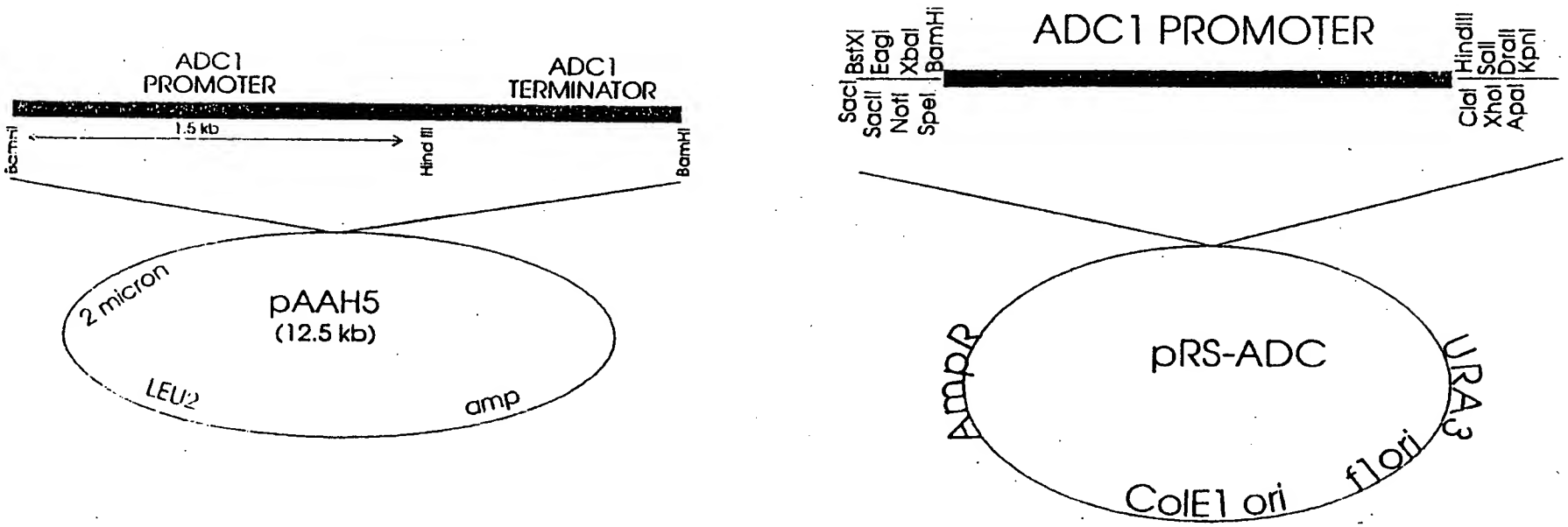

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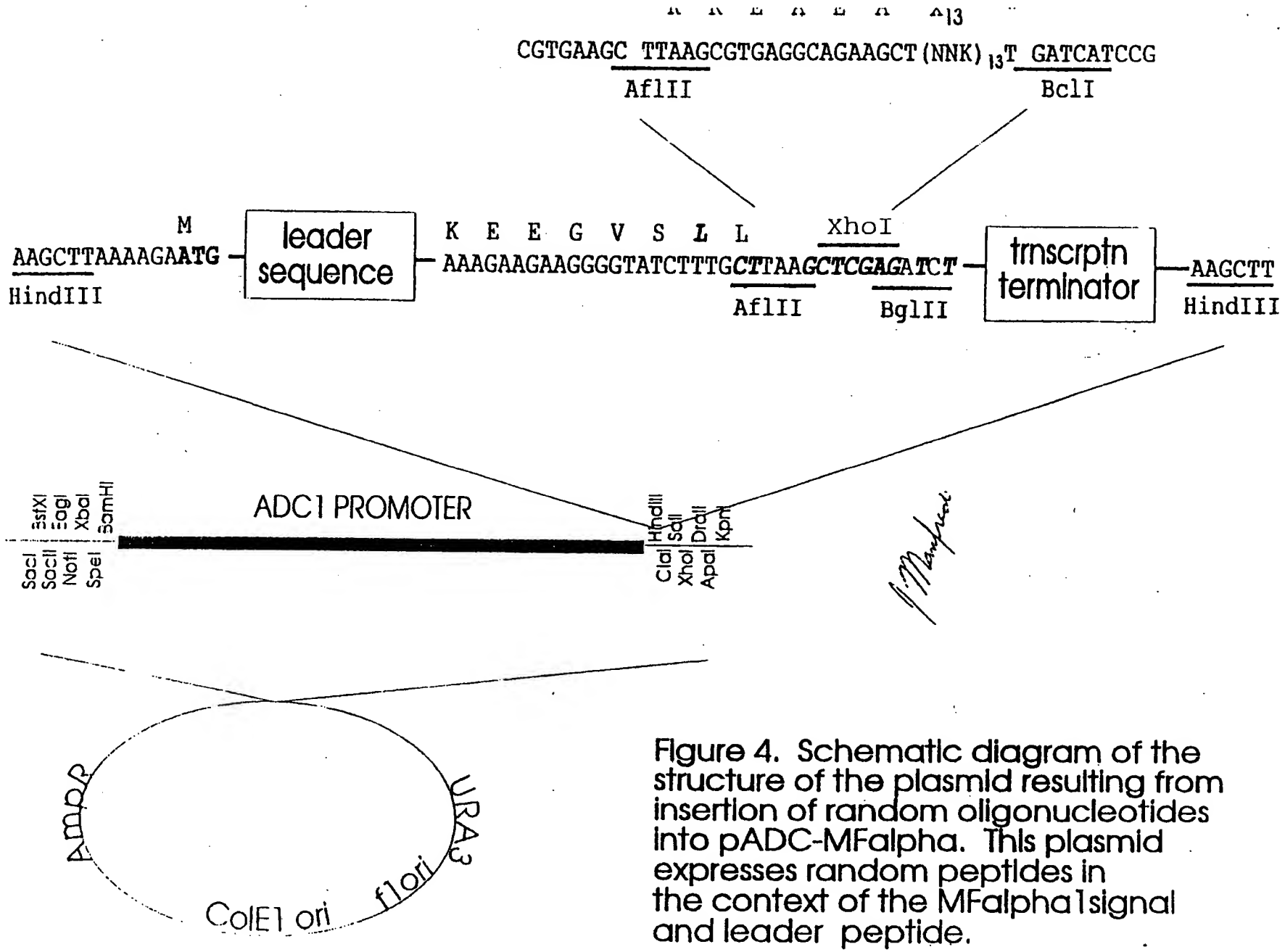

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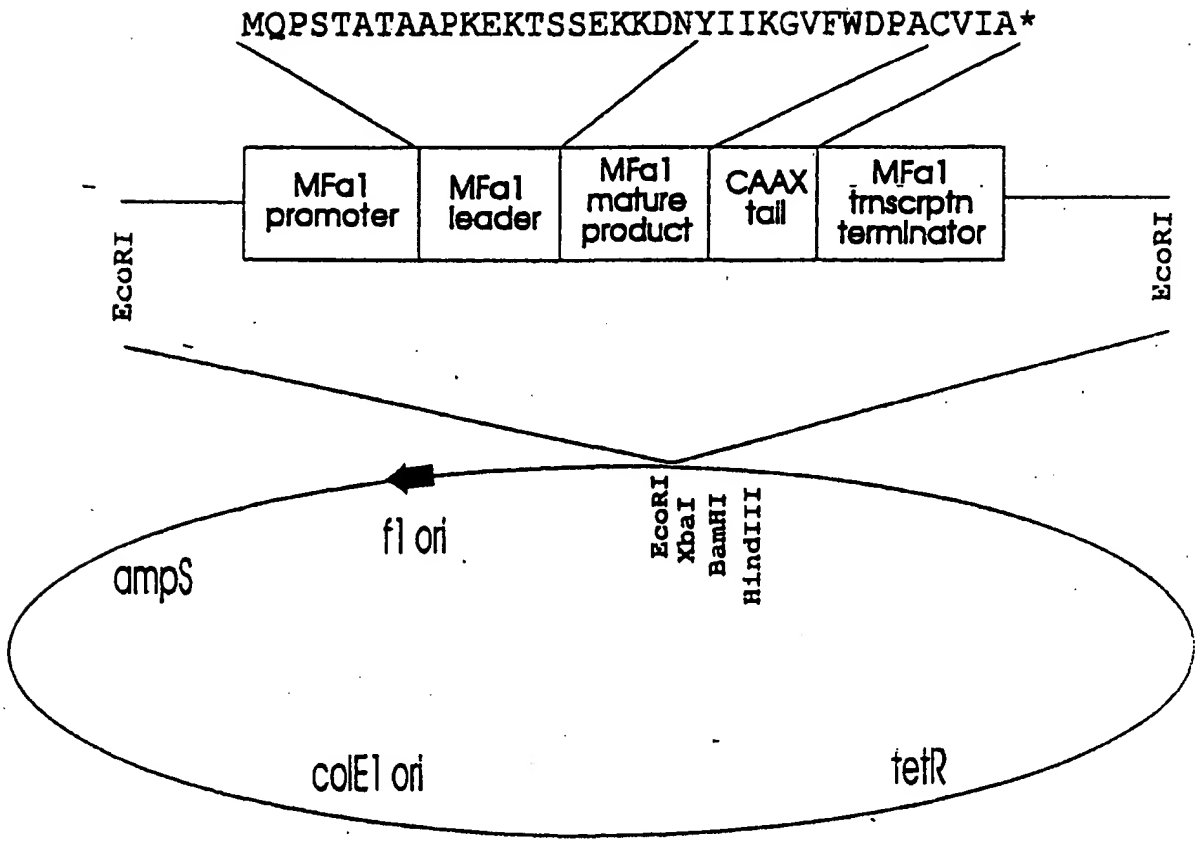

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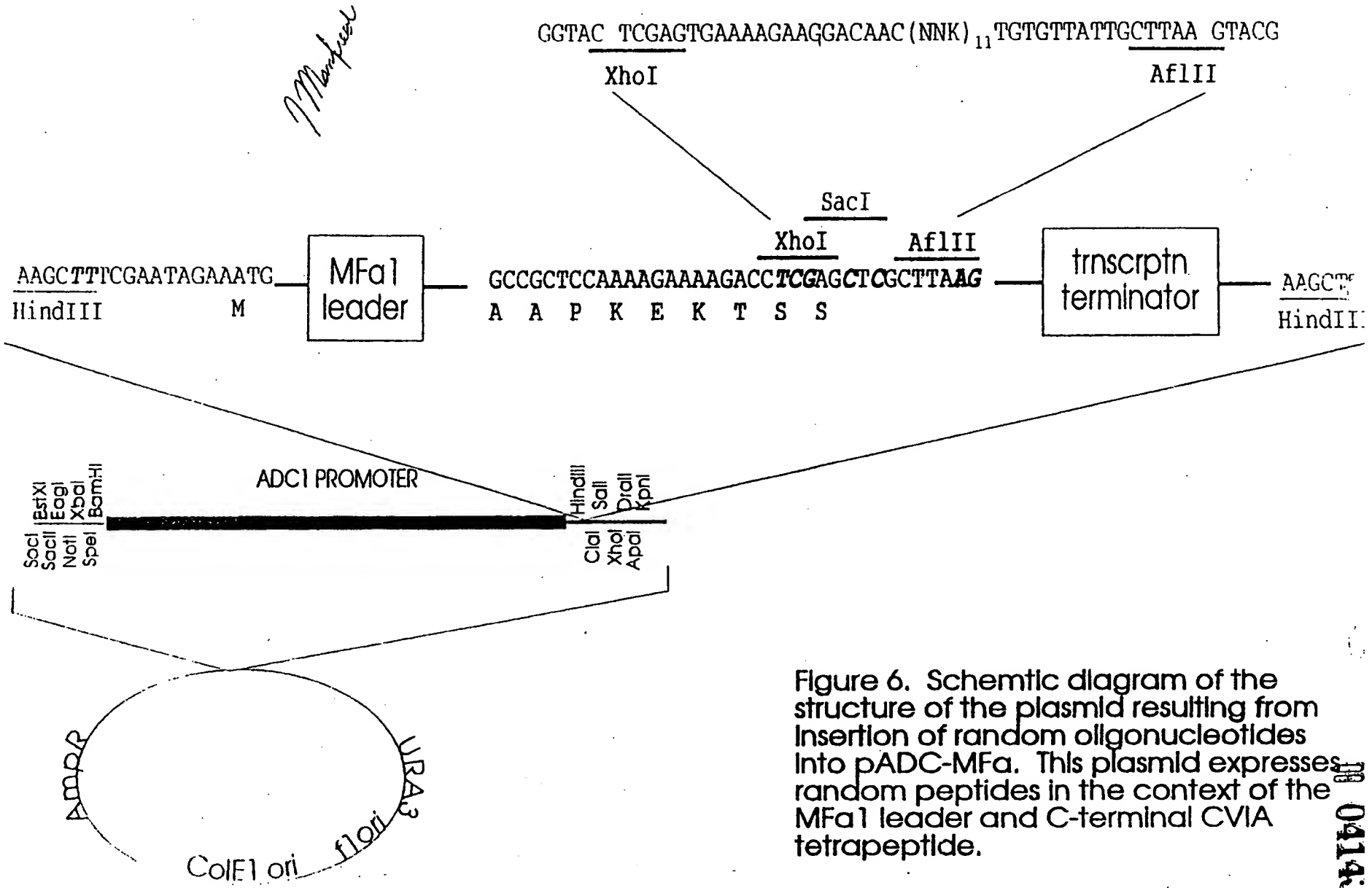

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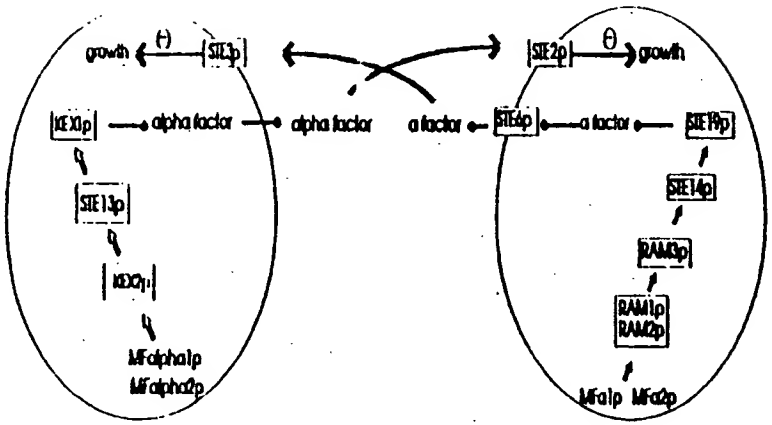
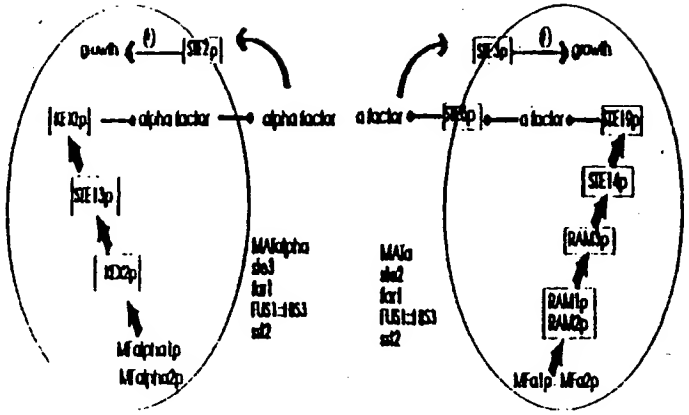
Synthesis, Release, and Targets of Mating Pheromones



Stage 1



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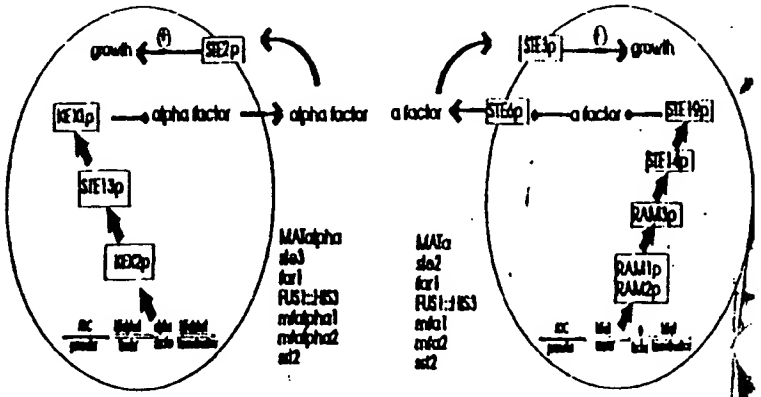


Stage 3

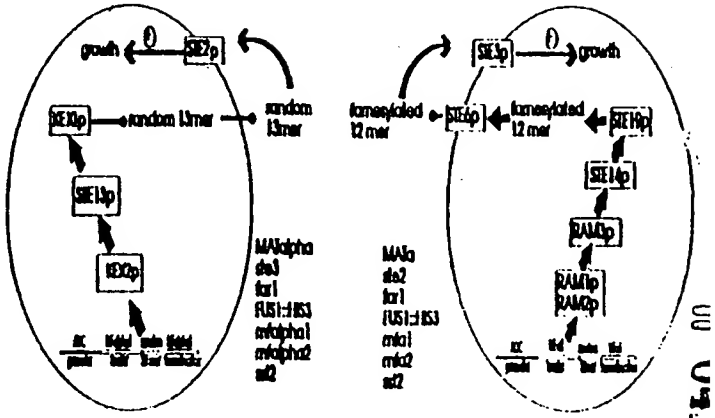
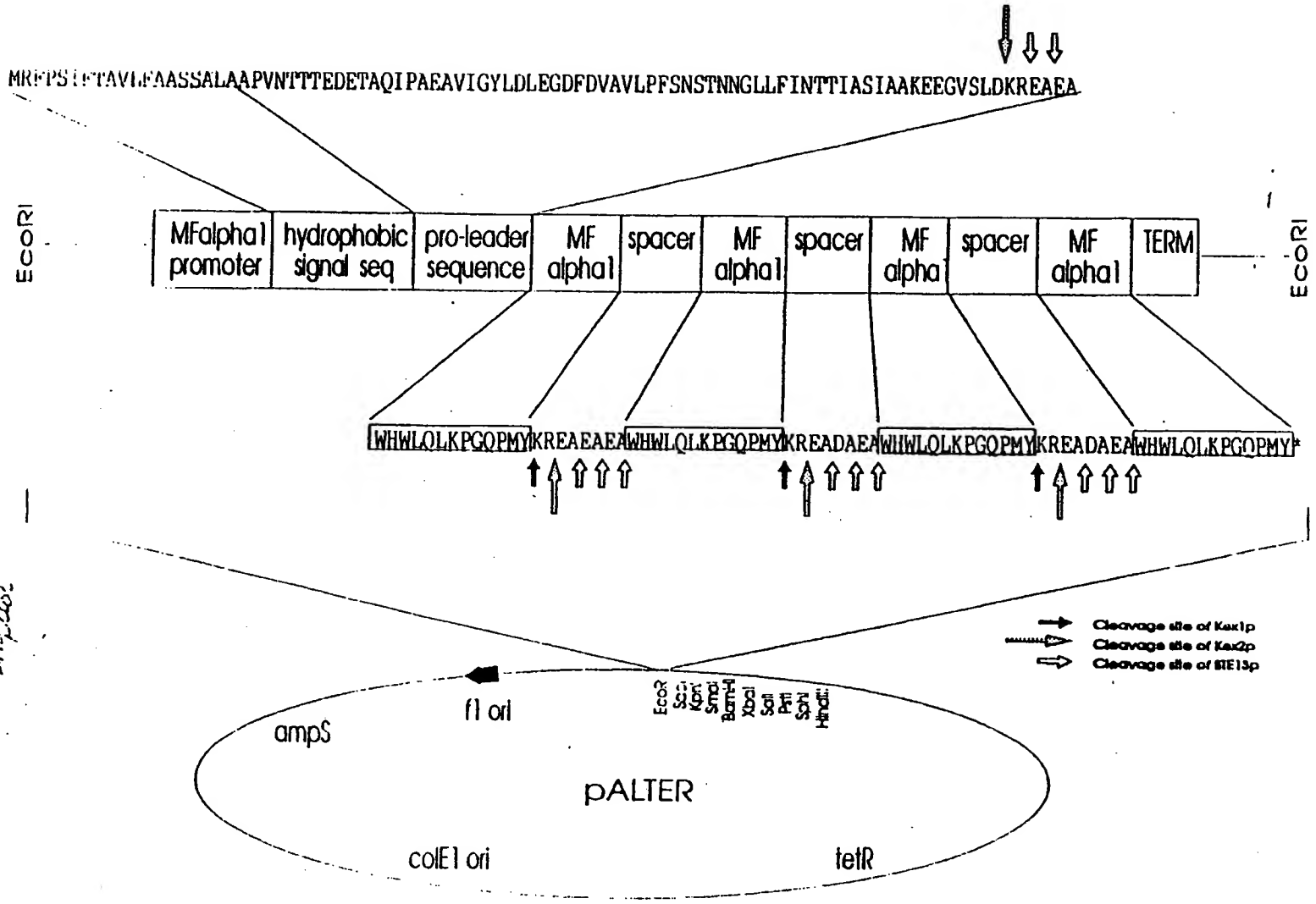


Figure 1



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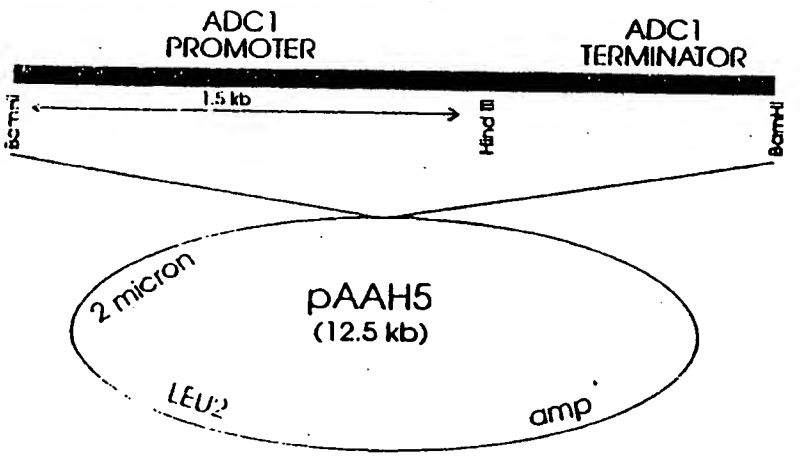

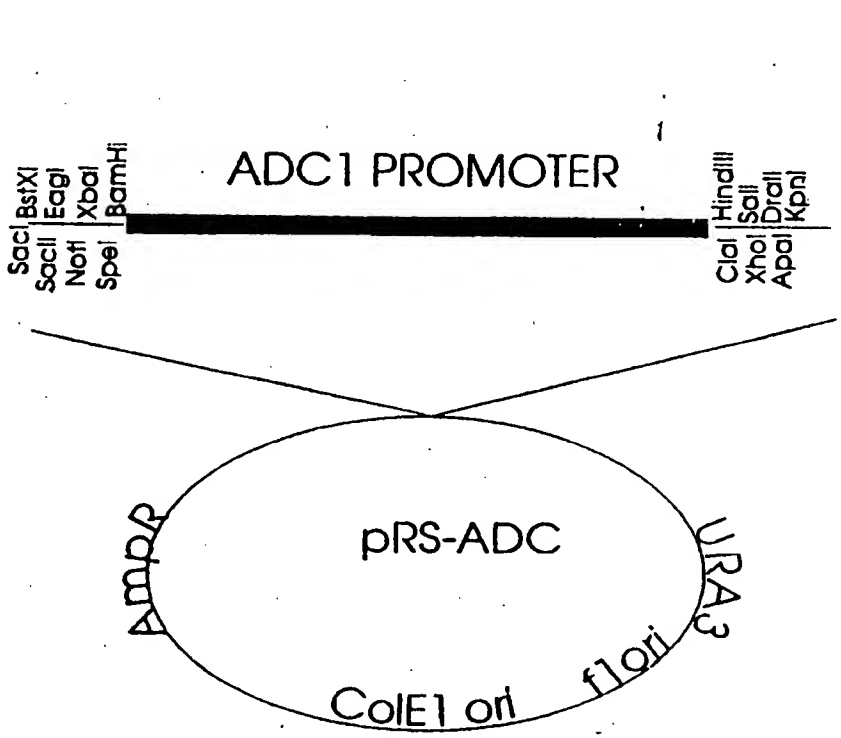

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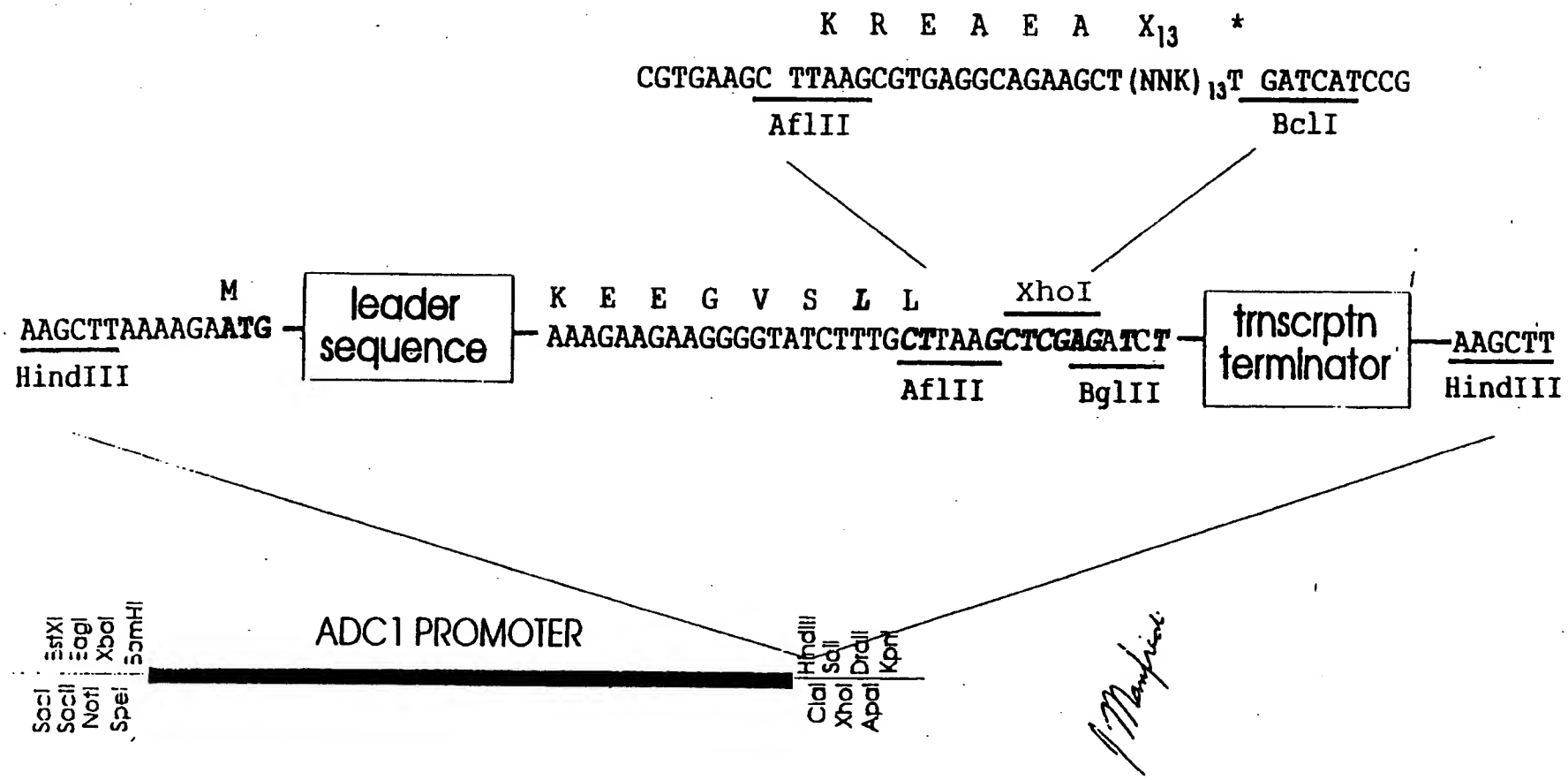

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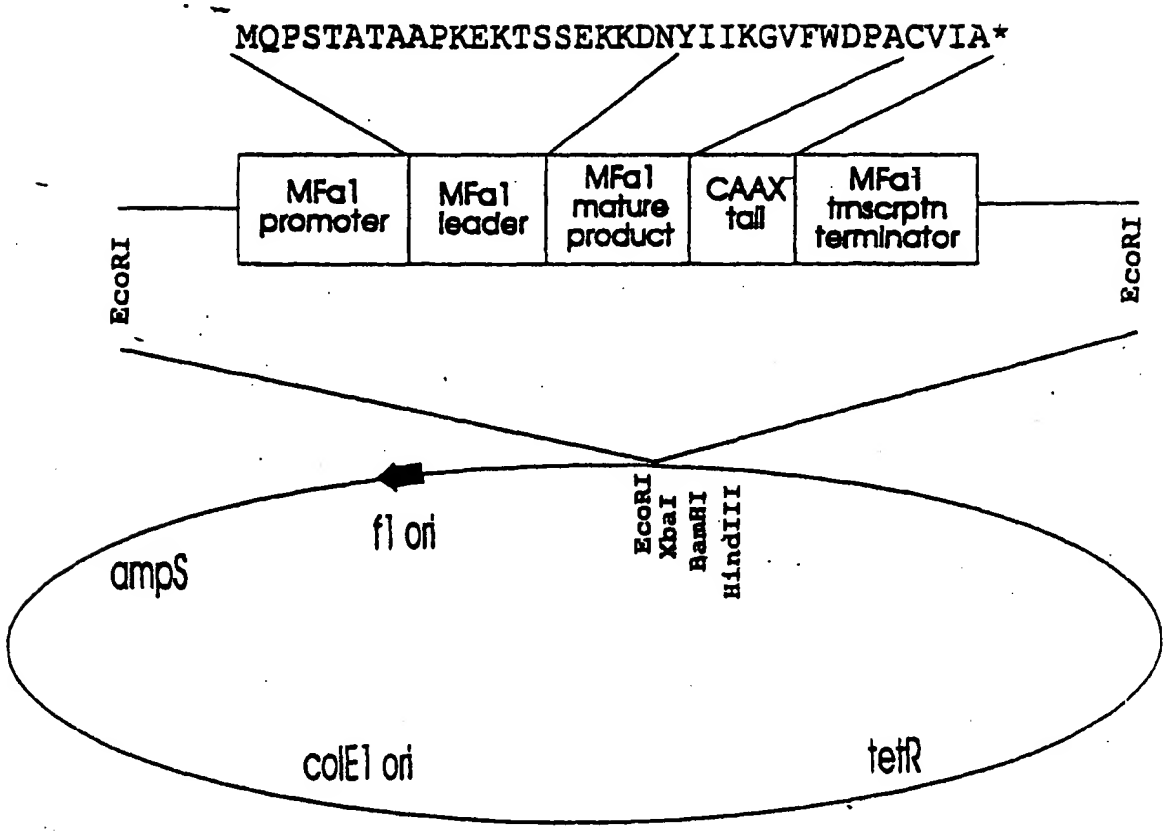

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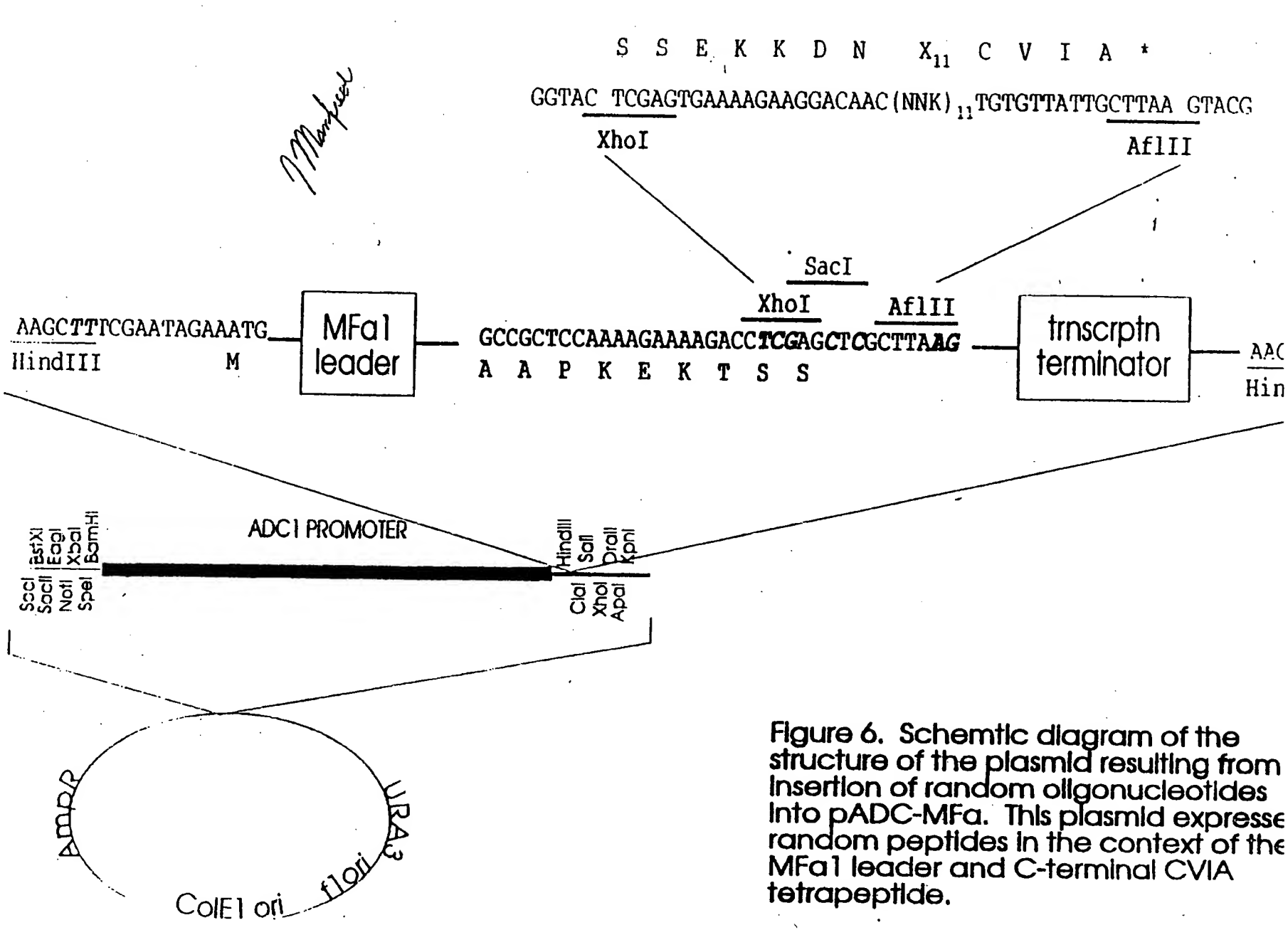

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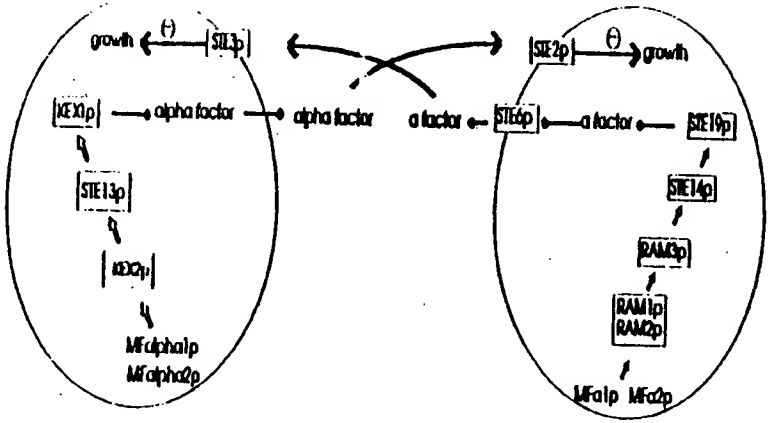
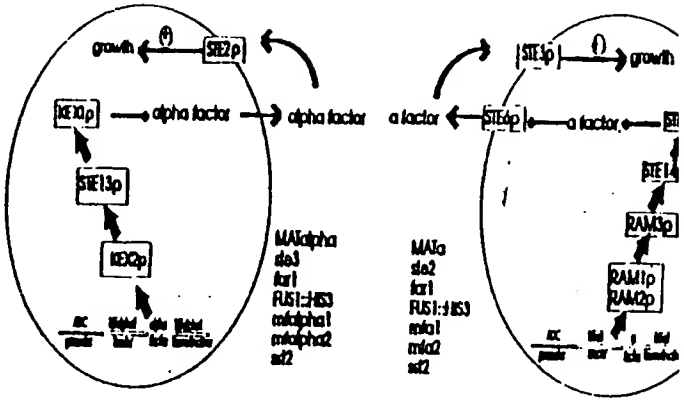
Synthesis, Release, and Targets of Mating Pheromones



Stage 2



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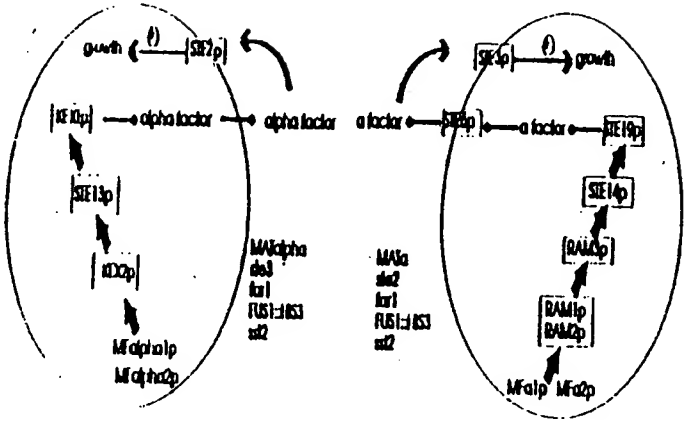

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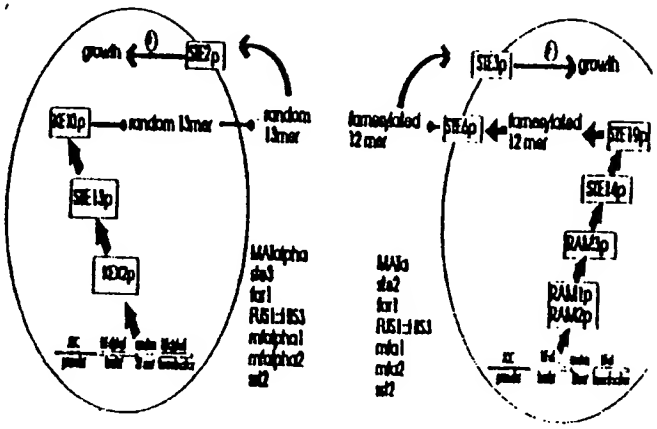

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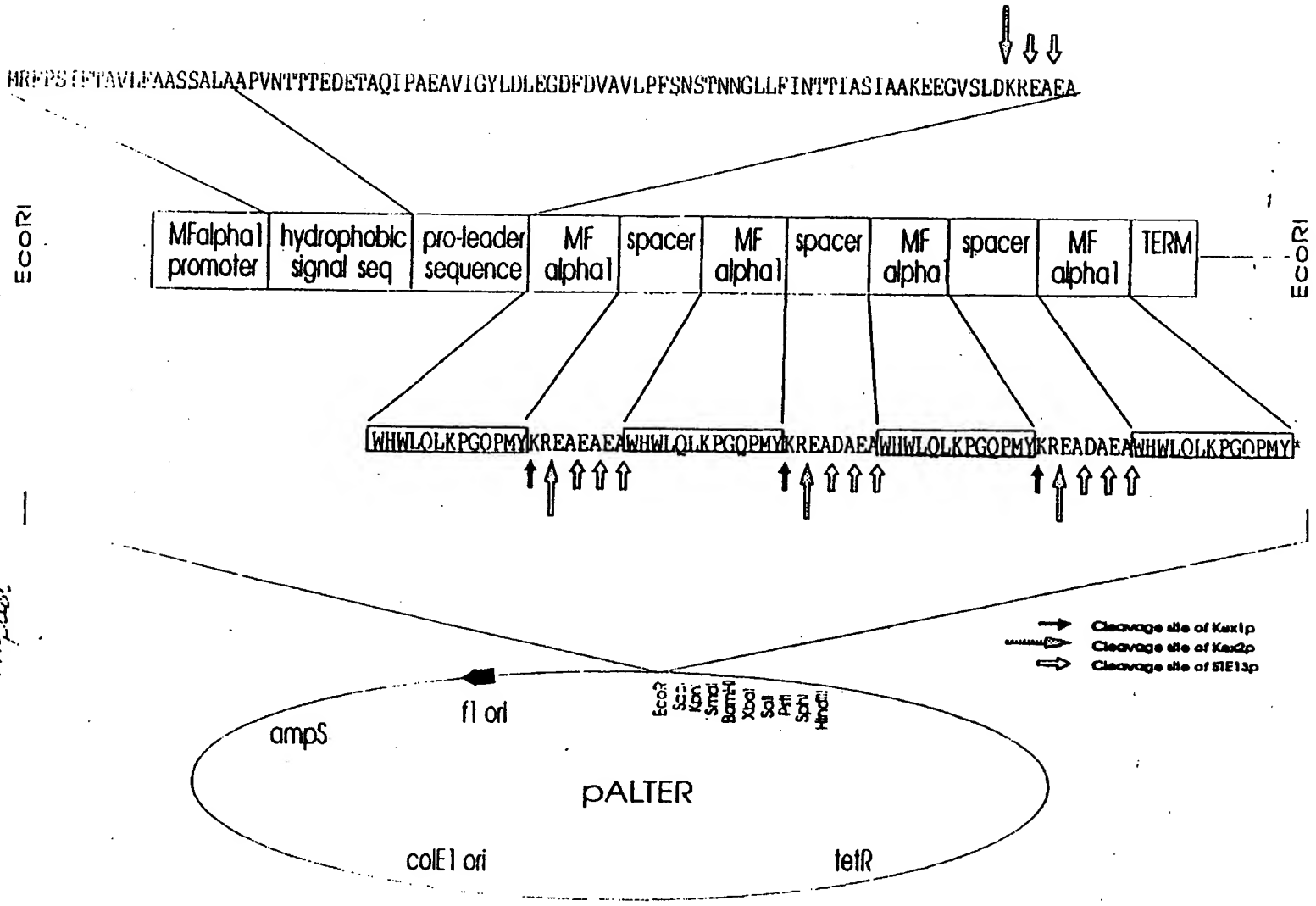

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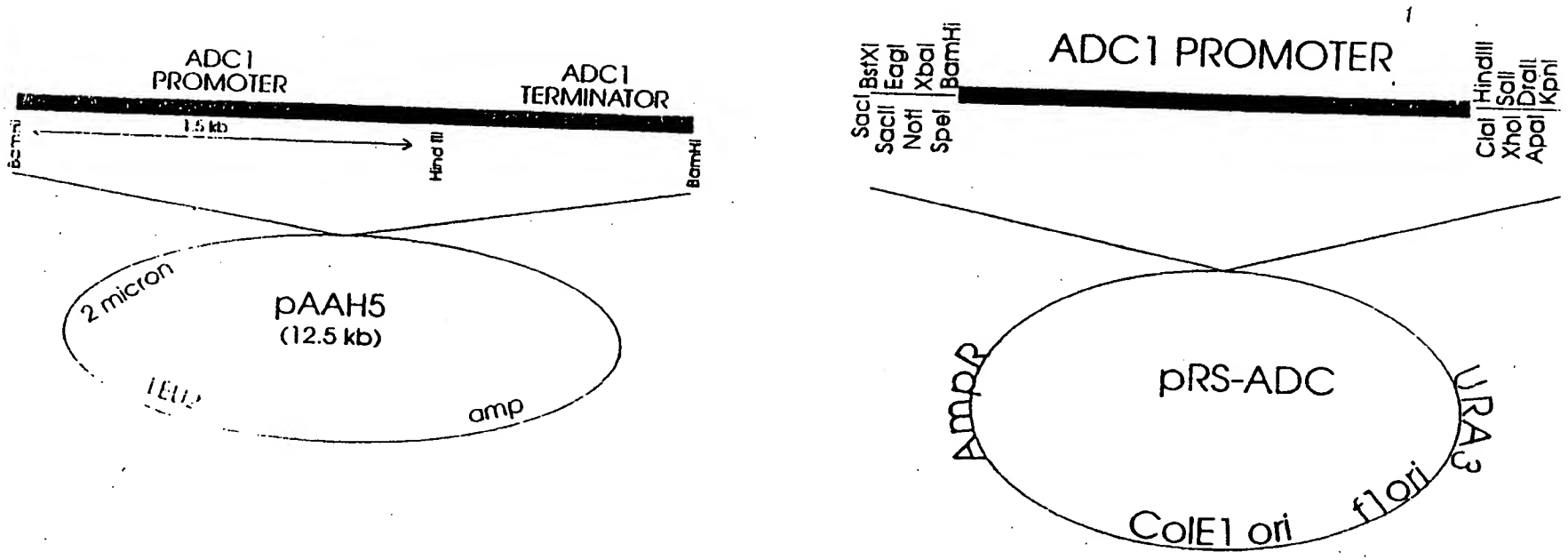

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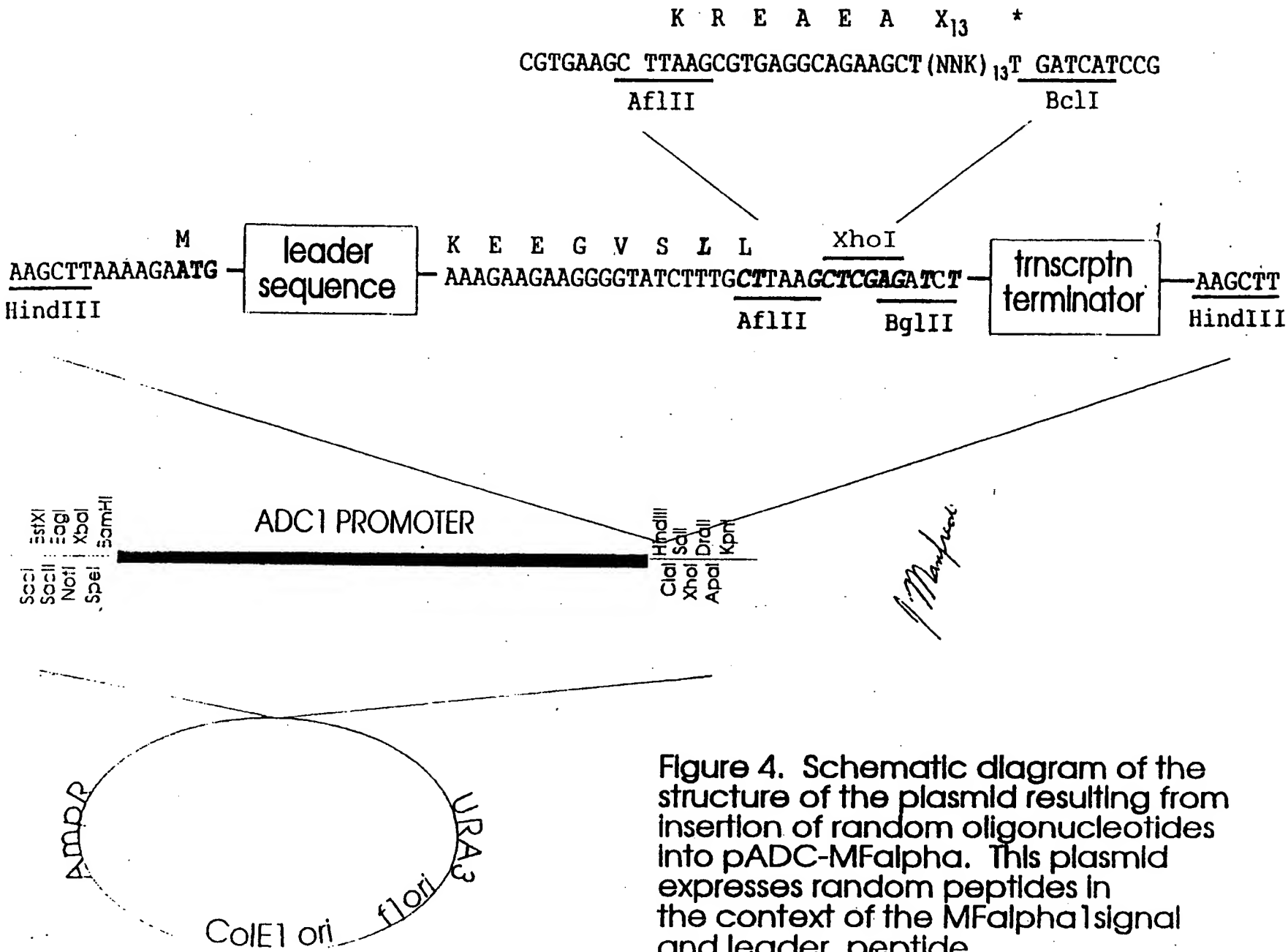

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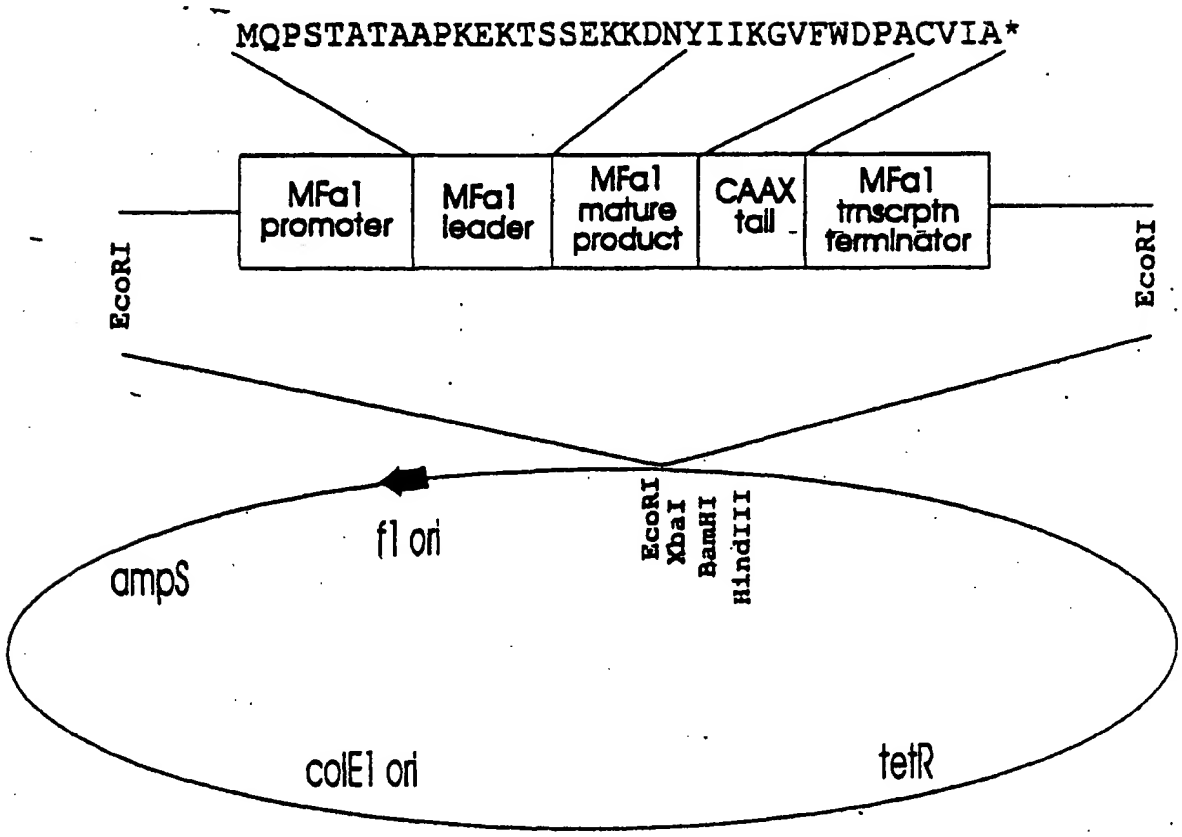

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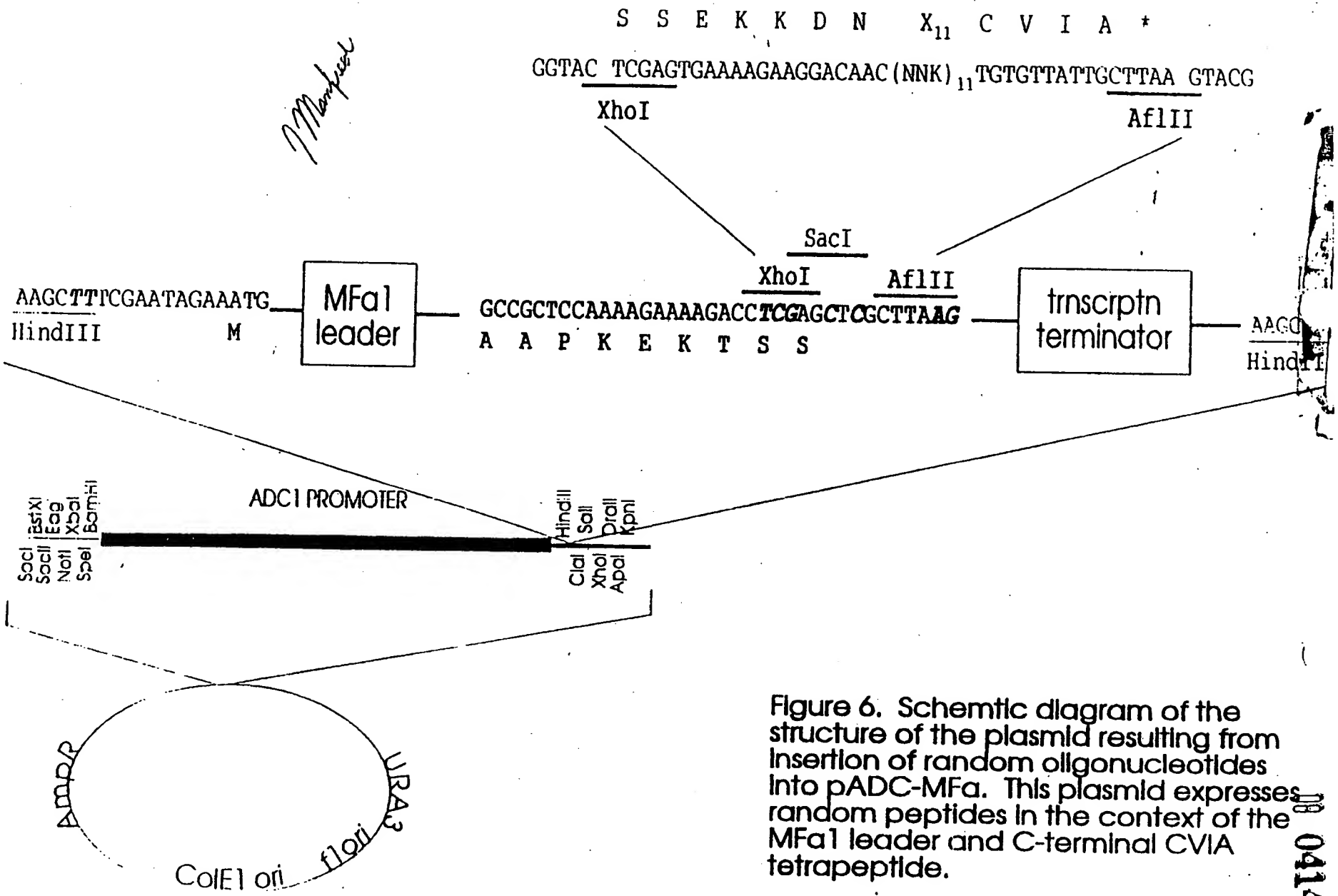

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